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(57) Abstract

The present invention relates to aqueous compositions of substances, such as organic molecules, which are useful to protect and preserve viable plant or animal cell membrane and tissue exposed to hypothermal and hyperthermal temperatures or non-physiological chemical conditions, and to modify the freezing process of liquids in biological plant or animal cells or tissue. More specifically, the present invention relates to the use of antifreeze polypeptide or antifreeze glycopeptide which is derived, for example, from the fluid or serum of Arctic and Antarctic fish. Preferred antifreeze compounds are related to those polypeptides having multiple alanine-alanine-threonine- or alanine-alanine-alanine-segments.

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COMPOSITION TO IMPROVE SURVIVAL OF BIOLOGICAL MATERIALS

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to aqueous compositions of substances, e.g. organic molecules, which are useful to modify the freezing process of liquids in biological tissue. More specifically, the present invention relates to the use of antifreeze protein or glycoprotein which is derived, for example, from the fluid or serum of Arctic and Antarctic fish. Preferred antifreeze compounds are related to those obtained from natural animal sources. More preferred are those polypeptides having multiple -alanine-alanine-threonine- or -alanine-alanine-alanine-segments. In some embodiments, a pendant sugar group is covalently attached to the threonine moiety.

A solution of antifreeze protein is perfused through animal or plant tissue, an animal organ or a whole living animal. The tissue, organ, or plant or animal is then carefully frozen to temperatures below -0.5°C and held at the low temperatures. The ice forms primarily along the c-axis of the ice crystal, and ice formation is inhibited in the direction of the a-axes (faces) of the ice crystal. This spicular ice growth compartmentalizes the concentration of the salts with the result that adjacent cells are not disrupted or completely dehydrated. The tissue, organ or animal is carefully thawed, and the tissue, organ or animal is functioning and viable. The preserved organs are particularly useful in transplantation therapy in a human being.

The present invention also relates to compositions of substances, e.g. organic molecules, which are useful to improve survival, functionality and/or structural integrity in biological materials, e.g., microorganisms, animal and plant cells, tissues, organs and whole plants or whole animals, exposed to temperatures and chemical environments different from their normal physiological temperatures and environments, by protecting at least the cell membranes from damage and cell contents from leakage due to exposure

to nonphysiological thermal and chemical conditions.

DESCRIPTION OF RELATED ART

The preservation of viable animal tissue, animal organs and living animals has been the subject of recent intense laboratory and medical research. Human organ transplants of heart, kidney, lung, liver and the like are now possible because of improved surgical techniques, improved anti-rejection drugs, and immediate availability of donated organs. Presently, donor organs are removed from a donor, cooled, stored on wet ice, but not frozen and within a maximum of a few hours are surgically placed in a recipient's body.

The preservation of animal tissue, animal organs and intact viable animals by freezing at lowered temperatures is presently limited to a few hours, because the normal formation of ice in an organ produces localized concentrated salt solutions. Water migrates from the nearby cells irreversibly dehydrating the cell. It is a major problem that these events disrupt the organ structure, and the organ does not reactivate upon thawing.

Advances in the development of immunosuppressants, improvements in organ transplantation techniques and the successful use of freezing for long-term preservation of cells have motivated intensive research efforts on methods for long-term preservation of biological organs through freezing. Recently, B. Rubinsky, U.S. Patent 4,531,373 disclosed an experimental technique using a directional solidification stage and low temperature scanning electron microscopy to facilitate the study the process of freezing in biological
tissues.

B. Rubinsky et al., (1988) Proceedings of the Royal Society London, B., Vol. 234, pp. 343-358), also describes experimental results and a mathematical model for the freezing process and the mechanism of damage in biological tissue and biological organs.

None of the available literature below disclose a composition or a method to preserve for long times frozen

tissue, organs or whole animals.

Earlier experimental results show that single, continuous ice crystals normally form along the blood vessels of frozen tissue. B. Rubinsky et al. (1988), Cryo-Letters, Vol. 8, p. 370; B. Rubinsky et al. (1988), Proc. Royal Soc. Lond., B234, 343. The structure of the frozen tissue depends on the cooling rate, (i.e., the temperature variation per unit time) during freezing. When tissue, such as liver, is frozen with low cooling rates (about 1°C/min to about 10°C/min), the smaller blood vessels (sinusoids) expand relative to those of the unfrozen normal liver tissue. In addition, the cells (hepatocytes) adjacent to the expanded sinusoids, are dehydrated without intracellular ice forming. However, at higher cooling rates, intracellular ice forms in the cells (hepatocytes) resulting in a reduced expansion of the sinusoids.

One explanation for the observed formation of continuous ice crystals along the blood vessels, for the expansion of the frozen blood vessels, and for the formation of intracellular ice during freezing with higher cooling rates is that ice formed in the vascular system does not propagate through the cell membranes or the blood vessel wall. Instead, ice forms within and propagates along the blood vessels where there is no barrier to the ice crystal growth process. Water in the cells surrounding the frozen blood vessels, being compartmentalized in small volumes, will, at first, remain supercooled. As the intravascular ice forms, water is removed from the solution in the vascular space, rendering the remaining solution hypertonic (higher in salts concentration). This higher concentration of solutes causes water to migrate irreversibly from the surrounding cells, through the semi-permeable cell membrane, into the blood vessel in order to equilibrate the difference in chemical potential. Consequently, the cells surrounding the blood vessel will dehydrate, and the water that leaves the cell then freezes in the vascular system. Water transport from cells through the cell membrane into the blood vessel, is a rate-governed

process, which depends on the permeability of the cell membrane. Therefore, when larger organs are frozen using higher (i.e. faster) cooling rates, sufficient water remains in the cell for intracellular ice to form prior to the complete dehydration of the cell. A more detailed description of the process of freezing and a mathematical model that supports this description is found in the Rubinsky, et al. (1988) reference above. This result also leads to the conclusion that one of the possible modes of damage to frozen tissue is the observed expansion of the blood vessels which causes the disruption of the structural (mechanical) integrity of the organ. This mode of damage apparently does not affect cells frozen in suspensions, and may explain why organs do not survive freezing under the same conditions in which cells in suspensions survive.

The normal patterns of ice formation, in which the energetically preferred direction of ice growth is also the a-axes (prism face) of the hexagonal prism ice crystal, governs the process of freezing in tissue. Any hexagonal prism facet of the a-axes of the three-dimensional ice crystal has the same energetic preference and, therefore, during freezing of tissue, the ice crystal can continuously follow and grow along the blood vessel. Furthermore, as discussed earlier, the large ice crystals of normal freezing do not incorporate solutes. This rejection of solutes results in more concentrated solutes, a mass transfer process and the irreversible water migration from local cells and tissue into the open vessel. This migration leads to the disruption of the structural integrity of the cells of the tissue or organ.

Additional background information can be found in:

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G.M. Fahy, et al. (1984) Cryobiology, Vol. 21 pp. 407-426.

W.F. Rall, et al. (1985) Nature, Vol. 313, pp. 573-575.

A. Trounson (1986) Fertility and Sterility, Vol. 46, pp. 1-12.

W.F. Rall, (1987) Cryobiology, Vol. 24, pp. 387-402.

J.M. Shaw, et al. (1989) Cryobiology, Vol. 26, pp. 413-421.

G.M. Fahy (1990), Scientific American, Vol. 262, pp. 20.

A. Arav, et al. (1990), Proc. 28th Annual Meeting Soc. for Cryobiology, Abstracts 42,43.

D. Turnbull (1969) Contemp. Phys. Vol. 10, pp. 473-488.

B. Rubinsky, U. S. Patent 4,531,373, July 1985.

B. Rubinsky, et al. (1985) Cryobiology, Vol. 22, pp. 55-62.

M. Mattioli, et al., (1988) Gamete Research, Vol. 21, pp. 223-232.

A. Arav, et al. (1988) Cryobiology, Vol. 25, pp. 567.

P. Quin, et al. (1982) J. Reprod. Fert., Vol. 66, pp. 161-168.

D. Pope, (1977) J. Anim. Sci., vol. 44, pp. 1036-1040.

All references, patents, articles, standards, etc. cited in this application are incorporated herein by reference in their entirety.

It would be extremely advantageous to have a composition and a method which would alter the preservation process of biological liquids in plant or animal cell tissue. Thus, when the frozen tissue, organ, plant or animal is carefully thawed, it results in viable cells, tissue, organ, plant or animal. The present invention provides such a preservation composition and method.

SUMMARY OF THE INVENTION

The present invention is related to a composition of biologically compatible substances, e.g., antifreeze peptide, useful to the survival, functionality, stability and structural integrity of biological materials, including proteins, enzymes, lipids, cell membranes, animal or plant

cells, microorganisms, tissues, organs, whole animals, or whole plants subjected to nonphysiological temperatures, either higher or lower than the normal physiological temperatures or to nonphysiological chemical environments, by interacting with the proteins, lipids and at least the cell membranes.

In other aspects, the present invention also relates to a composition of antifreeze protein useful in improving survival, functionality, stability and structural integrity of biological materials, including, proteins, enzymes, lipids, cell membranes, cells (animal or plant) microorganisms, tissues, organs, animals, or plants subjected to temperatures lower than 0°C in the presence of ice crystals:

(a) by modification of the structure of the ice crystals in the immediate vicinity of the proteins, enzymes, lipid or at least the plant or animal cell membranes;

(b) by reducing the number and the size of the ice crystals or by completely eliminating the ice crystals in the immediate vicinity of the proteins, enzymes, lipids or at least the cell membranes; or

(c) by modifying the mode in which solutes are rejected by the ice formation and thereby changing the chemical composition of the solution surrounding the proteins, enzymes, lipids or at least the cell membranes.

The present invention also relates to a composition useful to block ion channels in membranes to retard or prevent ion leakage (in general) and to stabilize cell membranes (in general), or in binding other macromolecules to proteins, lipids, or at least cell membranes.

The composition whose usefulness discussed above comprises at least one biologically compatible antifreeze substance, and a biologically compatible aqueous solution.

In one aspect the biologically compatible antifreeze substance is a macromolecule obtained from or substantially the same as a macromolecule derived from an animal selected from fish, amphibian, worm, insect or reptile, preferably

fish from Arctic, Antarctic, North Temperate or South Temperate Zones. More preferably, the protein is from body fluids (e.g. blood) from Antarctic fish, e.g. from the family Nototheniidae, including the species D. nawsoni and P. borchgrevinki or the Antarctic eel pout Rhigophila dearborni, or the Arctic winter flounder. All these antifreeze proteins are known and have the common property that they modify the structure of ice crystals.

In one embodiment, the biologically acceptable substance is selected from a polypeptide, a glycopeptide, a polypeptide covalently bonded to biologically acceptable carrier, a glycopolypeptide covalently bonded to a carrier or mixtures thereof.

In another aspect, the biologically compatible substance promotes ice crystal growth along the c-axis of the ice crystal, and inhibits growth of an ice crystal along the a-axis of the ice crystal. In another embodiment, the at least one type of biologically compatible macromolecule has alternating hydrophobic regions and hydrophilic regions which repeat between each 16-17 or 19-20 Angstroms, or preferably repeat about between about each 16.5 or 19.5 Angstroms.

In one embodiment, the aqueous composition further includes additional preserving, protecting or vitrifying compounds selected from glycerol, dimethylsulfoxide, ethylene glycol, polyvinylpyrrolidone, glucose, sucrose, propanediol, propylene, glycol, carboxymethyl cellulose, or mixtures of these compounds which are known to protect cells and biological materials against freezing damage or to promote vitrification.

The ability of the compounds to protect or stabilize membranes are also useful in the preservation of food; in cosmetics to restore, preserve or repair skin tissue; or in therapy for diseases associated with instability of cell membranes.

The ability to block ion channels is used in treating diseases associated with imbalances of the intracellular-extracellular ion transport.

The ability to attach to and interact with cell membrane, is used in attaching various macromolecules to the antifreeze proteins and thereby facilitating their attachment to the cell membrane.

In another aspect the present invention relates to a method for preservation, survival, functionality, stability and structure or integrity of biological materials, at non-physiological temperatures or in nonphysiological chemical compositions, including proteins, enzymes, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, whole animals or whole plants, which method comprises:

(a) bringing the moiety to be preserved in contact with a biologically acceptable substance in sufficient concentration to interact with all the proteins, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, whole animals or whole plants,

(b) exposure to the nonphysiological conditions;

(c) optionally first removing the macromolecule;

(d) returning the proteins, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, animals or plants, to a physiological temperature and composition, while optionally simultaneously removing the macromolecule; or optionally

(e) subsequently removing the macromolecule after returning the biological material to the physiological temperature and composition.

In one embodiment, the temperatures are hypothermic, i.e., close to 0°C or lower and are used for preservation of proteins, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, animals or plants. For example, pig oocytes are preserved in such a way at about 4°C to 24 hr or more. Rat livers are preserved by this method at 4°C for 24 hr or more.

In another aspect the present invention related to a method for preservation of proteins, enzymes, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, animals or plants at temperatures below

0°C to about 4K which method comprises:

(a) bringing the moiety to preserved in contact with the biologically compatible substance (e.g. AFP or AFGP in the presence of only an aqueous solution or with addition the other cryoprotective compounds such as glycerol, propylene glycol, etc.;

(b) cooling preferably to cryogenic temperatures (by such means as liquid nitrogen) and either vitrifying or freezing the system according to the various concentrations and cooling rates using higher concentrations of the additional compounds, such as propylene glycol or glycerol and high er cooling rates which lead to vitrification and to lower freezing temperatures (e.g. with 40% v/v propyleneglycol/water) and with a cooling rate of 1,750°C/min., vitrification is achieved);

(c) maintaining the proteins, lipids, cell membranes, cells (animal or plant), microorganisms, tissues, organs, animals or plants, at these temperatures for periods of up to 24 hours, 7 days, 52 weeks or more than 10 years,

(d) warming, by such means as warm fluids or microwave heating, to physiological conditions, and

(e) removing the biologically compatible substance, e.g. antifreeze glycoproteins and the other compounds, (e.g., by perfusion or flushing) and replacing them with physiological compatible solutions to regenerate the viable biological moiety.

For example, with 12.5% v/v propylene glycol/water at a cooling rate of 1,200°C/min., ice crystals were formed. In all cases, viable mouse embryos and pig oocytes were obtained after exposure to -130°C for several hours;

Pig oocytes, pig embryos and mouse embryos survive this protocol in a aqueous composition of 20 mg/ml antifreeze glycoproteins from Antarctic fish from the family Nothotheniidae.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B and 1C are transmission light micrographs of ice crystals (i), in aqueous solution is frozen with a cooling rate of 4°C/min on a directional

solidification stage, see U.S. Patent 4,531,373.

Figures 2A, 2B and 2C are scanning electron micrographs of liver tissue perfused with 40 mg/ml AFGPs (see definitions below) and frozen with a cooling rate of approximately 4000°C/min.

Figures 3A, 3B, 3C, 3D and 3E are scanning electron micrographs of frozen liver tissue.

Fig. 4 (4A to 4D) are photographs of the cryopreservation of immature pig oocytes.

Fig. 5 (5A to 5B) are photographs of the cryopreservation of pig embryos at the two-cell stage.

Fig. 6 (6A, 6B and 6C) are photographs concerned with cryopreservation of mouse embryos at the two-cell stage.

Fig. 7 (7A, 7B and 7C) are photographs showing with the hypothermic preservation of pig oocytes.

Figure 8 is a photographic representation of rat liver tissue (A7) at a magnification of about x 400. This tissue without AFGP treatment was cooled to -35°C at 21.5°C/min.

Figure 9 is a photographic representation of rat liver tissue at a magnification of about x 400. This liver tissue was flushed with a Krebs solution containing 20 mg/ml of AFGP fractions 1-8 (Table 1) at 37°C prior to cooling to -35° at 21.5°C/min.

Figure 10 is a graph of the bile production from whole rat liver treated with Krebs solution and Krebs solution AFGP as a function of time (see Example 7A).

Figure 11 is a graph of the LDH level from whole rat liver treated with Krebs solution and Krebs solution and AFGP as a function of time (see Example 7A).

Figure 12 in a graphic representation of the percentage of oocytes with normal membrane potential after hypothermic exposure for 4 hr at 4°C without and with various concentration AFGPs.

Figure 13 is a graphic representation of the percentage of oocytes with normal membrane potential after hypothermic exposure for 24 hr at 4°C without and with various concentrations of AFGPs.

As used herein:

"Abnormal nonphysiological chemical conditions" refers to conditions different from the normal physiological conditions include, but are not limited to high or lowered temperature, freezing, excess or limited carbon dioxide, excess or limited oxygen, excess or limited inorganic salts, excess or limited organic compounds, different pH values radiation or combinations thereof.

"Antifreeze proteins" or "antifreeze polypeptides" ("AFP") or "antifreeze glycoproteins" or "antifreeze glycopeptides" (AFGP)" are macromolecules found in the body fluids of some animals (e.g. cold blooded) which have the commonly known property that they reduce non-colligatively the phase transition temperature of water by direct interaction with and inhibition of the growth of ice crystal nucleii that form at temperatures below the phase transition temperature.

Antifreeze compounds are also known as "thermal hysteresis proteins" because while the phase transition temperature is apparently depressed during freezing by an amount much larger than the colligative effect of the molecule, it is not depressed during melting except to the extent caused by the colligative effect of the molecule. Prior to the present invention, this was the only known property of these antifreeze compounds. (Sources of antifreeze peptide (or protein) are described below).

"Cryogenic temperatures" refers in the area of cryobiology, below 0°C to as low as 4K or lower.

"Hyperthermic" refers to temperatures higher than the normal physiological temperature of a cell, tissue, organ, plant or animal.

"Hypothermic" refers to temperatures lower than the normal physiological temperature of a cell, tissue, organ, plant or animal.

"Optional" or "optionally" refers to the situation in which a component may or may not be present, or where a step may or may not be performed, within the scope of the invention.

invention.

"Prism planes" refer to another convention to describe the growing ice formation on an ice crystal. There exist secondary prism planes perpendicular to the a-axes and pyramidal planes that project off these planes. Crystallography terminology describes these planes in terms of the following pyramidal Miller-Bravais indices:

Primary prism plane	$(1\ 0\ \bar{1}\ 0)$
Secondary prism plane	$(1\ 1\ \bar{2}\ 0)$
Pyramidal pane from the primary prism plane	$(2\ 0\ \bar{2}\ \bar{1})$
Pyramidal plane from the secondary prism plane	$(1\ 1\ \bar{2}\ 1)$

Ice crystal growth under normal circumstances is along the a-axes. Ice crystal growth using the AFPs or AFGPs of the present invention is altered to be preferred in the direction of the c-axis.

For more information, see Peter V. Hobbs (1974) Ice Physics, Clarendon Press, Oxford, England, Appendix A etc., p. 725 ff.

"Rapid cooling" refers to a technique developed for long term preservation of cells and biological organs at cryogenic temperatures. The rapid cooling is used to produce very small, non-damaging ice crystals, see A. Trounson, (1986) Fertility and Sterility, Vol. 46, 1-12.

"Vitrification" refers to a technique for long term preservation of cells and biological organs at cryogenic temperatures. The technique involves introduction into the biological materials of different cryoprotective compounds such as glycerol, dimethylsulfoxide, propylene glycol, etc. which depress colligatively the phase transition temperature for water and increase its temperature. Next, the whole cell suspension or organ is rapidly cooled in the presence of the cryprotective compounds with the expectation that the water in the biological materials will remain polymorphous in a glass form and that no damaging ice crystals will occur. (See Fahy, G.M. et al.,

Cryobiology, Vol. 21, 407-426, (1984), W.F., Rall and Rahy, G.M. Nature, Vol. 313, 573-575, 1985)).

Sources of Antifreeze Proteins

Antifreeze proteins (AP-which includes AFP and AFGP) were found first in the body fluids of marine teleost fish which are hypoosomotic, have a blood serum freezing point of -0.7°C, but inhabit the polar ice-laden waters (Scholander et al. J. Cell Comp. Physiol., Vol. 49, 5-24, 1957). The first AP's were found by DeVries (Doctoral Thesis, Stanford, 1968) in Antarctic nototheniid fish. Two types of antifreeze proteins have been isolated from polar and temperate fish, glycopeptide and peptides. In studies of fishes with two exceptions, the antifreeze compounds are glycopeptides.

These antifreeze glycopeptides (glycoproteins) are present in eight distinct molecular weight classes ranging from - 2,500 to 34,000. They generally consist of a peptide backbone made-up of repeats of the tripeptide alanyl-alanyl-threonyl (the small glycopeptide may replace some alanines with proline beginning at position 7, with the disaccharide sugar beta-D-galactopyanosyl-(1→3)-2-acetamide-2-deoxy-alpha-D galactopyranose attached via a glycoside linkage to the hydroxyl side chain of each threonine (A. DeVries, Science, Vol. 172, 1152-1155, 1971).

These polypeptide or glycopolypeptides are available from a number of natural sources, e.g.: from body fluids of reptiles (e.g. turtles), invertebrates, insects, amphibians or fish. Preferably, the AFP's are obtained from the serum or body fluids of Arctic, Antarctic, North Temperate or South Temperate fish. More preferably, the serum and fluids of Arctic or Antarctic fish are used, e.g. See Table 1 below.

TABLE 1

MOLECULAR WEIGHTS OF ANTIFREEZE GLYCOPEPTIDES

A. Antifreeze glycopeptide isolated from the Antarctic nototheniidae are fish; Pagothenia (Trematomus) borchgrevinki.

Antifreeze Glycopeptide Fraction No.	Molecular Weight (Approx.)
1	33,700
2	28,800
3	21,500
4	17,000
5	10,500
6	7,900
7	3,500
8	2,600

Glycopeptide from Dissostichus mawsoni of structure

Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-
NAGA NAGA
GAL GAL
Thr-Ala-Ala-Thr-Ala-Ala-Thr-Ala-Ala-
NAGA NAGA NAGA
GAL GAL GAL

The molecular weights of the fractions are essentially identical to those of Table 1 above.

AFGPs isolated from the blood of Antarctic notothenid fish exist in at least 8 sizes depending on the number of repeats of the basic glycotripeptide unit, see Table 1. The molecular weight ranges between 2,600 and 33,700 (DeVries et al. (1970)) The AFGP's make up 3-4% of the blood of the fish and along with the sodium chloride, they lower the fish's freezing points below that of seawater. The AFTPs inhibit the growth of ice crystals by adsorption to the ice crystal (Raymond et al. (1977), DeVries (1984)). Adsorption occurs on specific faces of the ice crystal (primary prism place (1010)) resulting in inhibition of ice growth on these faces (DeVries, 1984), Consequently, in solutions of AFGPs, ice crystals grow predominantly on the

basal plane (parallel to the c-axis), to which the AFGPs do not absorb, and take the form of very small, needle-like ice crystals (Raymond et al., (1977); DeVries, 1988)). Peptide antifreezes can be found in several North Temperate, Arctic or Antarctic fish. The peptides vary in size and composition.

These polypeptide are essentially different lengths of the repeating tripeptide -alanine-alanine-threonine- where substantially each threonine is joined by a glycosidic linkage to the disaccharide B-D-galacto-pyransoyl-(1→3)- 2-acetamido-2-deoxy-alpha-galactopyranose.

The small glycopeptide may also have a small amount of proline located at positions 7, 10 and 13, but are otherwise structurally the same as the large glycopeptide.

Generally the higher the molecular weight, the more effective is the antifreeze glycopeptide in promoting ice crystal growth along the c-axis, for example, fractions 1-5 above individually or as a mixture as obtained by purification. Fractions 6, 7 and 8, having a lower molecular weight, individually or as a group are apparently less effective in preservation.

The antifreeze glycopeptides are essentially similar in all the Antarctic Nǫtotheniidae fish including (Pagothenia borchgrevinki, Trematomis Nicolia, Dissostichus Mawsoni (J.T. Eastman, and A.L.DeVries, A.L. Scientific American, Vol. 254, 106-114 (1986)). The same eight glycopeptides have also been isolated from northern hemisphere gadid, the rock cod (Gadus ogac) and in some other northern cods belonging to the family Gadidae (DeVries, A.L. Comp. Biochem. Physiol., Vol. 90B, No. 3, pp. 611-621 (1988). All the AFGP's isolated so far are similar in structure with relatively small changes such as the position occupied by proline in antifreeze glycopeptide 8 in northern species, or difference in size in northern cod, but essentially the same composition (A.L. DeVries, (1984) Phil. Trans. R. Socl. Lond., Vol. B 304, 575-588).

The other kind of antifreeze proteins found in fish are polypeptides. While the antifreeze glycopeptide are in

general polymers of a glycotripeptide unit Ala-Ala-Thr with disaccharide linked to the Thr side chain, the peptides are quite diverse structurally and vary in size and composition.

The antifreeze protein from the winter flounder, Pseudopleuronectes americanus, although having a specific activity similar to that of the larger molecular weight glycoproteins, lacks sugars entirely and instead has high percentages of hydrophilic amino acids (especially threonine and Asp) while still retaining a large amount (about 60 mol %) of alanine. The flounder protein primary structure has clusters of hydrophilic amino acids separated by sequences of alanine, (Duman and DeVries (1976) Comp. Biochem. Physiol, Vol. 533, 375-380).

Peptides from Winter Flounder

Asp-Thr-Ala-Ser-Asp-
Ala-Ala-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asp-
Ala-Ala-Ala-Ala-Ala-Ala-Leu-Thr-Ala-Ala-Asp-
Ala-Ala-Ala-Ala-Ala-Ala-Ala-Thr-Ala-Ala.

Origins of the Biologically Compatible Substance.

Peptides from the Antarctic eel pout Rhigophila dearborni

Peptide No.	Molecular Weight
1,2,3 (three components)	6,900

Asn-Lys-Ser-Val-Val-Ala-Asn-Gln-Leu-Ile-Pro-Ile-Asn-Thr-Ala-Leu-Thr-Leu-Ile-Met-Lys-Ala-Glu-Val-Val-Thr-Pro-Met-Gly-Ile-Pro-Ala-Glu-Asp-Ile-Pro-Arg-Ile-Ile-Gly-Met-Gln-Val-Asn-Arg-Ala-Val-Pro-Leu-Gly-Thr-Tyr-Leu-Met-Pro-Asp-Met-Val-Lys-Asn-Tyr-Glu-.

Other fish that produce antifreeze peptides are listed in A.L. DeVries, Phil. Froms, R. Soc. London, Vol. 304, 575-588 (1984) such as the Alaskan plaice, Atlantic sculpins, Grubby Sculpin (Yang, D.S.C. Nature, Vol. 333, 232-237, 1988) and the Antarctic Eelpout (Rhigophila dearborni). Recent reviews of the antifreeze proteins in fishes can be also found in (Feeney and Burchan (1986), Ann.Rev. Biophys, Biophys. Chem., Vol 15, 53-78,) and (Davies et al. (1988) Canadian J. Zool, Vol. 66, 2611-

2617).

V.S. Ananthanarayanan, Life Chemistry Reports, Vol. 7, pp. 1-32 (1989), also describes sources of antifreeze protein, particularly Type I, II and III.

These AFPs, AFGP's (or fractions and mixtures of fractions thereof) and others are available upon request from Dr. Arthur DeVries, Department of Physiology, Burrill Hall, 407 S. Goodwin, University of Illinois, Urbana, IL 61801.

These antifreeze proteins or peptides of the present invention may also be produced by synthetic means. These means include the use of a peptide synthesizer available commercially in the art as Model 430A, Applied Biosystems, Inc., Foster City, California. The operation manuals for this peptide synthesizer are useful. The synthesis procedures of J.J. Nestor, et al., U.S. Patent 4,318,905, and R.B. Merrifield, U.S. Patent 3,531,258 are specifically incorporated herein by reference and are adapted for the preparation of the Ala-Ala-Thr and Ala-Ala-Ala compounds described above. Once the peptide is prepared, the threonine residues are optionally bonded to the disaccharide by conventional methods.

The antifreeze protein of the present invention are independently selected from the protein themselves, or glycoprotein, or the protein or glycoprotein covalently bonded to a carrier such as biologically compatible antibody, gelatin, biocompatible polymer, peptide, sugar, or carbohydrate. Mixtures of these antifreeze materials are contemplated and are part of the present invention. Covalent bonding of a protein to a carrier by methods known to those of ordinary skill in the art are, for example, found in K. Rubenstein, et al., U.S. Patent 3,817,837, or M. Goodman et al., U.S. Patent 4,837,305, which are specifically incorporated herein by reference in their entirety.

Recombinant DNA Production of Antifreeze Polypeptide

It is also contemplated within this present invention to produce compositions wherein the peptides are produced

by recombinant DNA technology. The DNA sequences encoding these genes have been elucidated. See, for example, A.L. DeVries et al. (1971), J. Biol. Chem., Vol. 246, p. 305; Y. Lin, et al. (1972), Biochem. Biophys. Res. Commun., Vol. 46, p. 87; D.S.C. Yang et al. (1988), Nature, Vol. 333, p. 232; Y. Lin (1981), Proc. Natl. Acad. Sci. U.S.A, Vol. 78, p. 2825; P.L. Davies et al. (1982), J. Biol. Chem., Vol. 79, p. 335; B. Gourlie et al. (1984), J. Biol. Chem., Vol. 259, p. 14960; P.L. Davies et al., J. Biol. Chem., p. 9241; G.K. Scott et al. (1986), Can. J. Fish. Aquat. Sci., Vol. 43, p. 1028; G.K. Scott et al. (1988), J. Mol. Evol., Vol. 27, p. 29. Microinjection of the AFP gene into other species has been successful. See for example, Z. Zhu et al. (1985), Angew. Ichtoyol, Vol. 1, p. 31; Kexue Tongbao (1986), Vol. 31, p. 988; D. Chourrout et al. (1986), Aquaculture, Vol. 51, p. 143; R.A. Dunhan et al. (1987), Trans. Am. Fish. Soc., Vol. 116, p. 87; G.L. Fletcher et al. (1988), Can. J. Fish Aquat. Sci., Vol. 45, p. 352; N.D. Maclean et al. (1987), Bio Technology, Vol. 5, p. 257; G.W. Stuart et al. (1988), Development, Vol. 103, p. 403; T. McEvoy et al. (1988), Aquaculture, Vol. 68, p. 27; K. Ozato et al. (1986), Cell Differ., Vol. 19, p. 237; T.T. Chen et al. (1989), UCLA Symposium on Transgenic Animals; T.T. Chen et al. (1989), Aquaculture; P. Zhang et al. (1989), Mol. Reprod. Dev.; D.A. Powers et al. (1989), NIH Symposium on Transgenic Animals. The general formation of the DNA sequences to produce protein is found in the following U.S. Patents 4,237,224; 4,708,948; 4,376,071; 4,350,687; 4,444,760 and 4,722,998. The procedures are adapted to produce AFPs. All of these references are specifically incorporated herein by reference.

Recently antifreeze proteins (thermal hystersis protein) which is useful in the present invention were also found in many invertebrates. A list of these invertebrates is given in Table 2 and 3 with the references, found in the tables following immediately after the tables.

TABLE 2

Thermal Hystersis Protein Producing Invertebrates

A. Insects (minus beetles).

Order	Species	Reference
Collembola	7 spp.	Zettel, 1984
Plecoptera	Arcynopteryx compacta	Gehrken and Somme, 1987
Orthoptera	Parcoblata pennsylvanica	Duman, 1979
Hemiptera	Oncopeltus Fasciatus	Paterson et al. 1981
Mecoptera	Boreus westwoodi	Husby and Zacharissen, 1980
Lepidoptera	Choristoneura fumiferana	Hew et al., 1983

B. Coleoptera (Beetles)

Family	Species	Reference
Tenebrionidae	Tenebrio molitor	Ramsay, 1964 Patterson and Duman, 1978
	Meracantha contracta	Duman, 1977a
	Uloma impressa	Duman, 1979
	Platydema sp	Duman, 1979
Elateridae	Ampedus lineatus	Duman, 1979
	Ampedus sp	Duman, 1979
	Lepidotus discoideus	Duman, 1979
	Melanotus sp	Duman, 1979
Cucjidae	Cucujus clavipes	Duman, 1979
Pyrochridae	Dendroides canadensis	Duman, 1979, 1980

Lampyridae	Photinus sp	Duman et al., 1982
Coccinellidae	Coccinella novemnotata	Duman et al., 1982
Scolytidae	Ips acuminatus	Gehrken, 1984
Cerambycidae	Rhagium inquisitor	Bremdal and Zachariassen, 1988

C. Non-Inspect Arthropods

Animal	Species	Reference
Spiders	Philodromus sp	Duman, 1979
	Clubiona sp	Duman, 1979
	Bolyphantes index	Husby and Zachariassen, 1980
Centipede	Lithobius forficatus	Duman et al., 1982 Tursman and Duman, unpublished
Mite	Alaskozetes antarcticus	Block and Duman, 1989

D. Other Invertebrates.

Mussel	Mytilus edulis	Theede et al., 1976

TABLE 3

Amino Acid Compositions of Representative Insect THP's

(Values are in Mol %)

Amino Acid	Tenebrio Molitor -1[a]	T-4[b]	T-3[c]	Budworm[d]	Carudensis[d]
Asx	11.3	7.3	5.3	9.5	14.3
Thr	11.0	6.6	2.3	6.0	17.2
Ser	14.8	7.4	11.1	13.0	10.3
Glx	15.3	8.9	12.4	11.0	5.2
Pro	5.9	5.9	0.0	5.0	2.6
Gly	7.6	8.3	11.4	15.0	6.5
Ala	9.6	14.3	5.0	8.0	8.4
1/2Cys	0.0	0.0	28.0	6.0	15.9
Val	7.2	11.5	2.3	3.0	1.7
Met	0.0	4.8	0.0	0.0	0.2
Ile	3.3	7.1	1.0	1.2	1.5
Leu	3.9	0.0	2.2	6.5	1.9
Lys	4.8	6.8	15.4	3.1	3.4
Arg	1.1	2.6	0.0	8.0	4.8
Tyr	1.2	2.3	0.0	1.0	3.9
Phe	1.5	3.9	0.0	2.2	0.0
His	1.5	1.9	3.1	0.0	1.9
%Hydrophilics[f]	58.3	40.0	46.5	50.6	55.2

a Patterson and Duman, 1979

Table 3 continued

b Tomchaney et al., 1982

c Patterson and Duman, 1982

d Hew et al., 1981

e Wu and Duman, unpublished

f The percentage of amino acid residues with hydrophilic side chains (Asx, Glx, Lys, Arg, Ser, Thr), according to groupings of Manavalan and Ponnuswamy (1978).

References pertaining to Tables 2 and 3:

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L. Somme (1978) Norw. J. Ent., Vol. 25, pp. 187-188.

A.P. Tomchaney, et. al. (1982) Biochem., Vol. 21, pp. 716-721.

J. Zettel (1984) Rev. Ecol. Biol. Sol., Vol. 21, pp. 189.

GENERAL

In the present invention using aqueous antifreeze protein compositions, the process of ice freezing in tissue is changed, and the structural damage to the tissue reduced or eliminated through modification of the pattern of ice crystal growth. This advance is accomplished by modifying the pattern of ice crystal growth in tissue using novel compositions, e.g., peptides or glycopeptides from Arctic or Antarctic fish or other sources. The effect of the antifreeze proteins on the freezing pattern in aqueous solutions is documented extensively as cited above. The different antifreeze proteins from different sources adsorb to different crystal faces, however, all antifreeze protein inhibit ice crystal growth parallel to the a-axes, thermodynamically preferred direction of growth. Freezing in the presence of any kind of antifreeze protein invariably leads to ice crystals forming in the direction of the c-axis. The ice crystals grow in the form of spicules (spikelike structures). These small micron and

submicron scale spicular structures are stable and entrap between them the solutes that are rejected during formation of ice.

SOLUTION PREPARATION

The aqueous solution AFP or APGP compositions are prepared by any number of methods. Water (usually sterile) is contacted with AFP of AFGP and mixed to produce a solution of between about 0.1 and 100 mg/ml solute in water. Usually the antifreeze protein saturates in water in concentrations greater than about 100 mg/ml. Preferably, a solution of between about 1 and 60 mg AP/ml is produced, especically between about 20 to 40 mg/ml. The aqueous phase may also contain salts, sugars, ions nutrients (e.g. Krebs solution) and mixtures thereof in concentrations known in the art to be useful for preserving biological agents. The aqueous phase may also contain other materials, e.g. glycerol, etc., which are useful in the preservation of tissue, cell membrane, etc.

For use in organ transplantation and the like, sterile conditions and solutions must be used. The solutions may be prepared using sterile materials and sterile conditions. Also the solutions may be sterilized by methods known in the art, e.g. brief exposure to cobalt-60 radiation.

TISSUE PRESERVATION

To illustrate the effect of AFPs on the ice crystal structure, experimental results of the present invention are presented from earlier research in which the freezing pattern in a physiological saline solution is compared to the freezing pattern in a physiological solution with the addition of between about 1-100 mg/ml preferably about 40 mg/ml of antifreeze glycopeptides from Antarctic notothenii indae fish (Table 1). In this comparison, samples are frozen under controlled thermal conditions on a directional solidification stage. The directional solidification stage, described in greater detail in U.S. Patent 4,531,373 is an apparatus capable of freezing solutions or tissue samples with uniform cooling rates, between predetermined temperatures. The apparatus is used

in conjunction with a light microscope to produce results shown in Figures 1A, 1B and 1C which demonstrate the spicular growth in the presence of antifreeze glycoproteins.

One embodiment of the present invention is to perfuse solutions containing antifreeze proteins through the vasculature of an organ. Upon freezing, the ice crystals that form will be small, spicular and will entrap the solutes present. Consequently the cells will not be exposed to high saline concentrations, and the damaging expansion of the blood vessels will be eliminated. This effect will be demonstrated with detailed experimental results using the directional stage and scanning electron microscope in Figures 2A, 2B, 2C and 3A and 3B, 3C, 3D and 3E.

VITRIFICATION

It was formerly observed that cells, tissue or organs may not survive freezing with rapid cooling or "apparent vitrification". The expression "apparent vitrification" is used here to describe the observation that, at times, a solution is considered to be vitrified if it remains transparent after rapid cooling to cryogenic temperatures. However, the property of transparency is only an indication that the ice crystals are either too small or too few to reflect light and therefore, the vitrification is only apparent. In one aspect of the invention, it was expected that cells (or organs, tissue, animals) preserved by techniques in which the solution containing the cells (or organs, tissue, animals) is frozen by rapid cooling or apparent vitrification may be damaged by the preferential formation of very small ice crystals on the cell membrane, which may serve as a nucleation site. The antifreeze glycopeptides and peptides inhibit the growth of ice crystals and significantly reduce the size of the crystals formed by generating spicular ice structures. Therefore, these biologically compatible substances probably enhance the effectiveness of cryopreservation by preventing the formation of ice crystals on the cell membrane or by

reducing the size of these ice crystals.

The effectiveness of the antifreeze proteins (in vitrification) was evaluated on the cryopreservation of immature pig oocytes, two-cell stage pig embryos and mouse embryos, at the two-cell stage frozen by rapid cooling and "apparent vitrification." Pig oocytes and pig embryos at the two-cell stage were chosen because they present a very challenging model for which no successful cryopreservation has been heretofore achieved. In fact, pig oocytes and early-stage pig embryos usually cannot survive exposure to temperatures as high as 10°C for even brief time periods.

The probability of ice crystal nucleation during cooling is an inverse function of viscosity and temperature and a direct function of volume (D. Turnbull, 1969). In cryopreservation by rapid cooling, attempts are made to reduce the probability for nucleation by increasing the solution viscosity and by reducing the phase transition temperature through an increase in the concentration of various cryoprotectants. However, higher concentrations of cryoprotectants have a damaging effect on biological materials and, therefore, a proper balance must be found between a concentration that is sufficiently high to suppress nucleation and sufficiently low to avoid damaging the fragile cells.

These experiments were performed by exposing droplets of different size and composition to a variety of cooling rates on a special experimental system developed by B. Rubinsky, U.S. Patent 4,531,373. Rapid cooling, as well as rapid warming of samples, was performed using a Leitz Diaplan microscope to which a special directional stage was attached (A. Arav et al., 1990; B. Rubinsky, 1985; B. Rubinsky et al., 1985). The stage allows accurate control of cooling and warming rates between predetermined temperatures particularly as it is applied to vitrification and freezing by rapid cooling. A video camera was used in conjunction with the microscope to evaluate the morphology of the cells and the physical state of the solution.

An "apparent vitrification solution", (AVS) was useful

which contains 17.5% propylene glycol, (Fluka Chemicals, Switzerland), 2.5% glycerol (BDH Analar, England), 20% FCS (Fetal Calf Serum) (Gibco, Scotland) and 0.05 M sucrose in PBS (Dulbecco's phosphate buffered saline supplemented with 0.4 m/v BSA (Bovine Serum Albumin), 0.34 mM pyruvate, 5.5 mM glucose and 70 μg/ml kanamycin).

This solution is physiologically compatible with mouse and pig embryos and with pig oocytes. When 0.1 μl droplets of the AVS solution were cooled at the rate of 1,700° C/min (the highest rate possible with the directional solidification stage) to a temperature of -130° C (a temperature lower than the glass formation temperature for this solution) no ice crystals were observed through the microscope at 340x magnification. To illustrate the effect of volume and solute concentration, ice crystals were observed with all droplets of the AVS solution larger than 0.5 μl, and with all 0.1 μl droplets containing 12.5% propylene glycol and 2.5% glycerol when cooled at 1,700° C/min. No apparent devitrification, (that is, the formation of ice crystals was observed with droplets of the AVS solution larger than 0.5 μl, and with all 0.1 μl droplets containing 12.5% propylene glycol and 2.5% glycerol when cooled at 1,700° C/min.) No apparent devitrification (i.e., ice crystal formation) was observed when the samples were held at -130° C. However, devitrification was observed in some samples during warming to room temperatures even when the rate was as high as 1,700° C/min. The addition of AFGP's or AFP's was the AVS solution did not preclude the seldom and random occurrence of devitrification after "apparent vitrification". The AVS was the basic solution used in the experiment are reported to evaluate the effects of freezing with rapid cooling for used droplets larger than 0.5ml and for "apparent vitrification" droplets of 0.1ml. In the vitrification studies, only results from solutions that did not undergo devitrification were evaluated.

Evaluation of Cryoprotective Properties of AFPG & AFP in Oocytes and Embryos

To evaluate the cryoprotective properties of the AFGP's and the AFP's, immature pig oocytes, two-cell stage pig embryos and two-cell stage mouse embryos were introduced into either 0.1 ml droplets for vitrification, or droplets larger than 0.5 ml for freezing with rapid cooling of AVS with, and without, AFGP or AFP. These droplets were cooled on the directional stage under microscope observation at the rate of 1,700° C/min to -130° C. After 15 minutes at these temperatures, the samples were warmed at the rate of 1,700° C/min to room temperature. The survival of the embryos and oocytes was evaluated by in vitro culture followed by morphological and development analysis. Control experiments were performed by exposing embryos and oocytes to the different solutions in protocols identical to the rapid cooling experiments, but without cooling and warming, and evaluating their viability. The glycopeptides used in this work were obtained from Antarctic fish belonging to the family Nototheniidae (Dissostichus Mawsoni) (Table 1). A physiological composition was used which consists of one part of fraction 1 to 5 (high molecular weights) and two parts of fraction 7 and 8 (lower molecular weights) as obtained form A. DeVries, University of Illinois. Fractions 1-5 are obtianed as a mixture, and fraction 7-8 are obtained as a mixture. Experiments were performed with solution concentrations of 40 mg/ml glycopeptides. PBS is a standard buffered solution. This particular value was chosen because studies have shown that the depression of the freezing point of aqueous solutions of antifreeze glycoproteins is concentration-dependent and at these concentrations, it reaches saturation. A.L. DeVries, (1988).

After the cryoprotective properties of the AFGP's were established, parametric studies were performed with two-cell stage mouse embryos to determine the effect of concentration on the survival of the embryos. This animal

model was chosen for parametric experiments because it proved extremely sensitive to the effect of the glycopeptides. While no survival of embryos was achieved without the glycopeptides (0%), very high survival of embryos was obtained with the glycopeptides (82.5%, in vitro development to the blastocyst stage). The details of the parametric studies are listed in Table 4. The experimental procedures for pig oocytes and pig embryos are found in Example 4 below, and for mouse embryos is found in Example 5.

Table 4 lists the results of the experiments of Examples 3 and 4 below, starting with the pig oocytes, followed by pig embryos and mouse embryos. Table 4 also shows the solutions in which the embryos and the oocytes were tested.

TABLE 4: EFFECT OF AFGP ON OOCYTE VIABILITY

Time of Exposure Soln (hr)	PBS	PBS + 0.1 mg/ml AFGP 1-8	PBS + 1 mg/ml AFGP 1-8	PBS + 40 mg/ml AFGP 1-8	PBS + 40 mg/ml AFGP 1-5	PBS + 40 mg/ml AFGP 7,8
4	6/48 (12.5%)	7/25 (24%)	19/27 (70%)	54/70 (77%)	12/43 (28%)	11/47 (23%)
4	9/48 (18.75%)	11/29 (37%)	20/27 (74%)	59/70 (84%)	21/43 (48%)	18/47 (38%)
24	0/17 (0%)	0/14 (0%)	6/14 (42%)	7/17 (41%)	0/14 (0%)	0/13 (0%)
24	0/17 (0%)	0/14 (0%)	9/14 (64%)	9/17 (53%)	0/14 1(0%)	0/13 (0%)

CRITERIA A = $\frac{\text{number of cells with electrical potential} > |u| - |v|}{\text{total number of cells}}$

CRITERIA B = $\frac{\text{number of with electrical potential} > |u| - |2v|}{\text{total number of cells}}$

The protocol to which the cells were exposed is the one described earlier in which the embryos and oocytes were introduced in various solutions with some of the embryos and oocytes exposed to rapid cooling while others which did not undergo cooling, kept as controls for the solution effect. The results are presented, for pig oocytes, as the ratio between the number of oocytes which reached the MI or MII stage after in vitro maturation, and the total number of oocytes exposed to the experimental protocol. For the pig embryos, it is the ratio between the number of embryos that reached the four-cell stage after in vitro development and the total number of embryos exposed to the experimental protocol. For the mouse embryos, it is the ratio between the number of embryos that reached the blastocyst stage over the total number of embryos exposed to the experimental protocol. The numbers in the bracket give the ratio expressed as percentage.

The experiments with pig oocytes, pig embryos, and mouse embryos exposed to the AVS solution, show that this solution does not have a damaging effect. However, when the embryos and the oocytes were cooled rapidly or vitrified to cryogenic temperatures in the AVS solution, not a single embryo or oocyte survived. These results demonstrate that the damage to these cells is a consequence of cooling and exposure to cryogenic temperatures. Microscopic examination revealed that a primary site of damage following rapid cooling in the AVS solution was the oolemma in the case of oocytes and the blastomer membrane for embryos which did not retain integrity as illustrated in the Figures, especially in Figs. 4B, 5A, and 6B. However, in the presence of the glycopeptides the cells that were rapidly frozen or vitrified retained viability as shown in Table 4.

In Figures 12 and 13 are shown the membrane potential for oocytes at 4 and 24 hr at 4°C. The dramatic retained membrane potential viability at concentrations of 1-40 mg/ml of AFGPs is found in Figure 13. Figures 12 and 13 values are mean ± one standard deviation. Each exp. group

consists of 5 oocytes and n represents the number of groups.

In particular, as described in experiment 3, the cell membrane was protected by the glycopeptides.

UTILITY

It is apparent from the disclosure herein that the AFP and AFPG aqueous composition of the present invention is useful in cell preservation, membrane preservation, tissue preservation, organ preservation or in whole plant or whole animal preservation.

In general, the antifreeze proteins have the property that they noncolligatively lower the apparent freezing point of aqueous solution resulting in a freezing temperature that is lower than the melting temperature. They also have the general property that they inhibit or restrict growth on different facets of ice crystals while allowing the growth along the c-axis. Until now it was not know that these proteins can be also use to interact with other proteins, and, in particular, protein on the surface of cell membrane and to protect the structural integrity of the membrane and stop leakage through the membrane and block ion channels. This is the first time that these properties and its applications are observed and are described as part of this invention.

EFFECT OF THE AFGP'S ON THE CELL MEMBRANE

Initially, the effect of the antifreeze proteins on modification of ice crystal growth focused on the use of this property in preservation of cells, tissue, organs and whole animals at temperatures below freezing. However, in studies described above and experiments such as Examples 1 and 4 (also Table 4), in which the morphology of cell membranes was evaluated, it appeared consistently that the antifreeze proteins provide complete protection to the morphology of the membrane and its structural integrity. Therefore, a procedure was developed to determine if the antifreeze proteins protect by interacting directly with cell membranes, and contacting the protein directly to cell membranes.

Pig oocytes were chosen as the experimental model in this study because these oocytes are temperature sensitive and cannot survive exposure to hypothermic temperatures as high as 10°C, i.e., temperatures that are higher than the phase transition temperature. Therefore, an experiment was designed in which the effect of the AFGP's on the cell was studied at temperatures higher than the phase transition temperature, but lower than the normal body temperatures. If a protective effect of the AFGP's is found, it is probably not directly related to the ability of the compound to modify ice crystal morphology or inhibit of ice crystal formation.

The oocytes were introduced in different solutions of standard buffer PBS solution with antifreeze glycoproteins (Fractions 1-5, Table 1) from fish of the family Nototheniidae. They were kept in a constant temperature environment for various periods of time and then the membrane potential was measured. The structural integrity was also determined by microscope evaluation.

To establish a criteria for an intact oolema, preliminary experiments were performed for each batch of oocytes in which the membrane potential of the fresh oocytes was measured at 22°C. The mean value of the electrical potential, u, and the standard deviation, v, were calculated for each batch. The mean and the standard deviation were measured in fresh oocytes in a buffer solution and in a buffer solution with 40 mg/ml antifreeze glycopeptides Fractions 1-8 (Table 1) as obtained from A. DeVries, supra.

Table 4 above summarizes the results derived from measuring the resting potential across the oolemma. Table 4 gives the ratio between the number of oocytes considered to have an intact oolema relative to the number of oocytes used for each experimental condition, (the number in the brackets is the ratio in percentage,) for different concentrations of the AFGP's and different times at 40°C

The comparison shows that the glycopeptides have little effect on the resting potential of each oocyte. To

determine the integrity of the oolema, two statistical criteria were established, one less stringent than the other. The oolema in an oocyte was considered to be intact if the absolute value of the measured resting potential difference, was higher than the absolute value of either $|u|-|v|$ or $|u|-|2v|$.

The results from evaluating the structural integrity of the oolema are consistent with the electrical potential measurement and are illustrated by Figures 7A, 7B, and 7C. The results clearly show that the membrane is preserved morphologically intact in the presence of the glycopeptides. Furthermore, ion leakage that is probably the most prevalent cause of damage during hypothermic exposure is significantly inhibited in the presence of the antifreeze proteins. This implies that the antifreeze proteins have the ability to protect cell membranes at hypothermic temperatures and to block ion channels. The evidence of the use of this new discovery in hypothermic preservation of cells and organs are given below in Examples 6 and 7, respectively.

It is emphasized that prior to this research it was not known that antifreeze proteins have the useful properties of preserving cell membranes and blocking ion channels.

WHOLE ORGAN PRESERVATION

Cryopreservation of a whole organ, e.g. liver from a mammal, such as a rat, is described in Examples 2, 7 and 7A below. The organ is surgically removed, held in a preservation solution at 20-37°C, preferably 24°C. A major blood vessel is cannulated. The well-known Langendorf perfusion system (with a first bottle containing, for example, Krebs solution and antifreeze polypeptides in a 1mg/ml to 100 mg/ml) is used. See, for example, D.E. Pegg et al. (1986), Cryobiology, Vol. 23, pp. 150-160.

A second bottle of solution contains a physiologically compatible saline solution and appropriate quantities of glycerol, dimethyl sulfoxide, ethylene glycol, polyvinyl pyrrolidone, glucose etc. or mixtures of these substances

which are known as protectants for cells of biological origin.

These two bottles of solutions are connected to a mixing valve having known adjustable flow rates (e.g. 0.1 to 10 ml/min, preferably about 5 ml/min) and a computer to accurately vary the flow rate and mixing of each bottle's contents immediately prior to perfusion. The perfusion using the solutions of bottles 1 and 2 is well known in the art as described by D.E. Pegg et al. (1988) above for kidney and G.N. Alink et al. (1976), Cryobiology, Vol. 13, pp. 295-304; (1977) Cryobiology, Vol. 14, pp. 409-417 and 399-408; and (1978) Cryobiology, Vol. 15, pp. 44-58, and K.E.F. Hobbs et al. (1969), Cryobiology, Vol. 6, pp. 239-245 for heart. The Krebs solution is perfused through the organ held at about 20-37°C at a rate of about 4 ml/min.

The mixing switch provides intermediate amounts of Krebs solution and glycerol solution in pulses of time lengths controlled by the computer, for example, 0.01 sec. to 0.1 sec. The two solutions mix in the delivery tube or in a special mixing chamber.

The antifreeze protein/Krebs solution is initially adjusted so that at the end of the perfusion process a concentration of between 1mg/ml to 40 mg/ml is achieved in blood vessel space in the tissue. The majority of the AFPs are found within the vascular space (bed) of the organ (not within the cells of the liver or the blood vessels). The AFPs ("AFGP's") are usually too high in molecular weight to significantly penetrate the cell membrane. The organ, e.g. liver, is next placed in a cooling stage as described in U.S. Patent 4,531,373, and the temperature of the whole perfused organ is then cooled at a rate of 1°C per minute to -32°C or to -70° or until -150°C is reached. The organ is then cooled as needed using a liquid nitrogen to -196°C or in liquid helium to 4K and held at this temperature for an indefinite time (e.g. 72 hr). The frozen organ is then carefully thawed by immersion in a cold or warm liquid, e.g. water or saline, at a rate of between about 0.1 to 10°C per min. (preferably about 1°C per min.) using known

techniques up to 37°C maximum. Alternatively, carefully controlled microwave heating is used to thaw the perfused organ, e.g. liver. When the thawed organ (liver) reaches about 0°C, the nutrient solution of Krebs is perfused through the large cannulated blood vessel. When warmed to about 20 to 37°C, preferably 37°C, the thawed organ recovers not only cell function, but also organ function. Preserved tissue samples are taken as needed.

A systematic study of the effects of the AFGP on rat liver cold-storage was done to compare control storage solutions and solutions containing the AFGP (see Example 7A). The results obtained are compared for three different storage periods, 6, 12 and 24 hr. The functional tests include the production of bile and enzymatic activities of lactic dehydrogenase (LDH). The Krebs solution is selected as the control solution. The reason for this selection is to separate the protective qualities of other storage solutions from the effects of the AFGP.

Useful mammalian organs include liver, kidney, heart, brain, lung, pancreas, spleen, ovary, stomach and the like. The organ of a mammal, such as a human being, is preferred.

PRESERVATION OF A WHOLE MAMMAL

The cryopreservation of a whole mammal, e.g. rat, human, is adapted from Examples 1, 2, 7 and 7A below. An adult mammal, e.g. rat, is anesthetized and the carotid vein or aorta is cannulated.

The computer controlled mixing switch provides intermediate amounts of Solution 1 (Krebs/antifreeze protein), and solution 2 (glycerol/saline solution) bursts of time lengths controlled by the computer, for example 0.01 sec. to 0.1 sec. to 1 sec. time lengths. The two solutions mix in the delivery tube or optionally in specially provided mixing chamber.

A Krebs solution is first perfused briefly through the mammal. Heparin about 1,000 to 3,000 units is added to inhibit blood clotting.

A solution known as a cryopreservative for cells, such as glycerol in saline, is perfused through the organ using

the mixing switch arrangement described herein.

Optionally, blood substitutes such as the University of Wisconsin solution or Euro-Collins solution are added to the perfusion solution. The rat is then perfused with a solution of antifreeze polypeptide (e.g. Example 1) at a rate of 0.1 to 10 ml/min for 20 min. The body temperature of the mammal is lowered to less than 2°C. The mammal is then cooled from the exterior to the interior at a rate of 0.1 to 10°C/min.

The mammal is then cooled to -80°C or to -150°C or to 4K and held at this temperature for an indefinite period, e.g. 7 days or 7 months. The frozen mammal is then carefully thawed at a rate of about 0.1 to 10°C/min to about 0°C in the presence of oxygen/nitrogen (20/80; v/v). A nutrient blood substitute, e.g. heparin, (about 1,000 units) at 0°C is perfused through the mammal via the carotid vein, the mammal is warmed to its normal biological temperature. Tissue function, organ function and whole animal function is recovered.

PRESERVATION OF CELLS AND TISSUE AT TEMPERATURES BELOW FREEZING

The cryopreservation is demonstrated in cells, e.g. human ooytes, pig oocytes, embryos, human or leucocytes, platelets, e.g. pancreatic islets, hepatocyles, corneas, skin. See examples 4 and 5. Various cryoprotective agents such as glycerol, propylene glycol are introduced in the cell together with the antifreeze proteins essentially as described in Examples 3, 4 and 5. The different solutions of cryoprotective agents are chosen to either produce freezing or vitrification such as 5M propylene glycol. The cells or tissues are then cooled rapidly to either produce freezing or vitrification with cooling rates of e.g. 1750 °C/min or as high as required to temperatures of -130°C to -180°C, or to 4K and held at that temperatures for an indefinite period. The cells or tissue are then carefully thawed. Cell function and tissue function is recovered.

PRESERVATION OF ORGANS BY RAPID FREEZING VITRIFICATION

The procedure is the same as described in the whole

organ preservation section except that the concentration of the cryoprotectant is taken to a high level, such as 5M propyleneglycol, and the cooling rates are high enough, such as 1,750°C/min, to produce either rapid freezing or vitrification as desired as opposed to slow freezing in the earlier application. The use of antifreeze proteins is essential for the successful preservation of organ tissue by vitrification.

PRESERVATION OF WHOLE MAMMALS
BY RAPID FREEZING OR VITRIFICATION

Again, the procedure is substantially identical for preservation of whole mammals by slow freezing except that high concentrations of cryoprotective agents are used 5M propylene glycol and high cooling rates, e.g. 1750°C/min, the perfusion with antifreeze glycoprotein is essential for preservation. The examples are found in the text below.

HYPOTHERMIC PRESERVATION OF CELLS

The procedure of Example 6 is followed except that liver cells are first contacted with aqueous AFGP solution. These cells survive the cooling and are viable upon careful warming to physiological temperatures.

HYPOTHERMIC PRESERVATION OF ORGANS IN COLD STORAGE

The procedure of Example 6 is followed except that an organ, e.g., liver or heart is contacted with the aqueous AFGP solution. This organ survives the cooling and is viable upon careful warming to physiological temperatures.

HYPOTERMIC PRESERVATION OF ORGAN
BY CONTINUOUS PERFUSION

Example 7 is repeated except that the blood containing the antifreeze glycoprotein is continuously perfused through the organ.

HYPOTHERMIC PRESERVATION OF WHOLE MAMMALS
BY COLD STORAGE

The procedure of Example 7 or 7A is followed, except that the perfusion is applied to a whole mammal.

HYPOTHERMIC PRESERVATION OF
WHOLE MAMMALS BY CONTINUING PERFUSION

This embodiment is essentially identical to the above

preservation except that a blood substitute containing the antifreeze protein is also continuously perfused through the animal.

HYPOTHERMIC PRESERVATION OF CELLS, TISSUE, ORGANS, MAMMALS

Example 6 is repeated with the exception that antifreeze proteins are brought in contact with cells, tissue, organs, mammals where it is desired to protect them from hyperthermic damage.

PRESERVATION OF CELLS, TISSUE (SKIN), ORGANS, MAMMALS FROM A CHEMICAL ENVIRONMENT THAT IS NOT OPTIMAL

Example 6 conditions are repeated with the exception that a non physiological chemical environment such as high carbon dioxide is present.

PRESERVATION OF CELL MEMBRANES

Cell membranes are brought into contact with physiologically capatible solutions with antifreeze proteins.

BLOCKING ION CHANNELS

Cell membranes are brought into contact with physiologically compatible solutions with antifreeze proteins. In channels, for example, sodium and postassium are found to be substantially blocked.

ATTACHMENT THROUGH ANTIFREEZE PROTEINS

Various macromolecules are artificially attached to antifreeze protein and than introduced in cell suspension, tissue, organs, or whole mammals. The antifreeze protein attach then to cell membranes and thereby bring molecules in the vicinity of the cell membrane.

DETAILED DESCRIPTION OF THE FIGURES

The following is a detailed description of the Figures:

Figures 1A, 1B and 1C

Figure 1A shows the frozen region 11 (i) and the planar solid-liquid interface in a physiological saline solution at the onset of the normal freezing process.

Figure 1B shows the final dendritic, finger-like structure of ice 12 (i), during freezing of a physiological saline solution. Figure 1C shows spicular structure of ice

crystals 13 (i), during freezing of a physiological saline solution with 40 mg/ml AFPs. The scale bar 14 shown in Figures 1A, 1B and 1C is 50 micrometer.

Figures 1A, 1B and 1C show the ice crystal morphology in aqueous solutions frozen with a cooling rate of 4° C/min on the directional solidification stage. Figures 1A and 1B show a well-known sequence of events during freezing of saline solutions. In saline solutions, ice forms and grows on the prism plane of the ice crystal, forming wide macroscopically smooth surfaces (Fig. 1A). During freezing, the ice rejects the solute which accumulates at the ice-water interface. The increased concentration of salts causes a colligative decrease in the change of phase temperature on the interface and lead, through the well-known phenomenon of constitutive supercooling instability, to the transformation of the ice crystal morphology from a planar structure to a dendritic one as shown in Figure 1B. However, Figure 1C shows that the ice crystal growth pattern in the presence of antifreeze glycoproteins (40mg/-ml) is very different. Figure 1C shows spicular (spike-like) ice crystals much smaller in dimensions from the dendritic ice crystals seen in Figure 1B. The antifreeze glycoproteins ice structure is spicular from the onset of the freezing process. Using polarized light, it is shown that the spicular ice crystals grow in the direction of the c-axis. The small spicular ice crystals incorporate between them, the AFPs and the other solutes in the solution. Figures 1A, 1B and 1C show that the incorporation of the solutes stabilizes the spicular ice growth along the c-axis. It is observed that in the presence of antifreeze glycoproteins the ice crystals are very small. The saline solution is incorporated between the submicron size ice spicules. Therefore, the salt solution does not concentrate significantly to produce a significant change in chemical potential. As a result, in organs water does not migrate from the surrounding cells dehydrating, expanding the blood vessels, and collapsing these cells.

Figures 2A, 2B and 2C

Figures 2A and 2B have black 21 and white arrows 22 which point toward longitudinally and transversely sectioned sinusoids 23 (s), which show spicular ice crystals. All the spicular ice crystals are oriented in the same general direction. Structurally intact hepatocytes surround the sinusoids. The round nucleus is evident in some of the cells, where it is marked with a white dot 24. Rounded ice crystals are observed in all the cells.

Figure 2C shows higher magnification of the spicular ice structures in a large blood vessel 25 (bv). The margin of the blood vessel is shown with black arrows 26. Typical ice crystals in adjacent cells are marked with white circles 27. The scale bar 28 for Figures 2A, 2B and 2C is 10 micrometer.

Figures 3A, 3B, 3C, 3D and 3E

Figure 3A shows normal liver tissue frozen with a cooling rate of 4°C/min on a directional solidification stage, as described hereinabove. Continuous, smooth ice crystals are seen inside expanded sinusoids, (s). The adjacent hepatocytes (h) are dehydrated.

Figures 3B and 3C shows liver perfused with antifreeze glycopeptides (40 mg/ml), which are similar to those in Table 1 and frozen with a cooling rate of 4°C/min on a directional solidification stage. The cross-section through a large blood vessel 31 (bv) shows spicular ice crystals 31 confined within the blood vessel 33. (The surface of the blood vessel is marred by debris formed during the fracture.) The outline of box-like hepatocytes 34 (h) fractured along the cell membrane is marked by arrows 35. The dimensions and the shape of the hepatocytes are typical to that of normal hepatocytes.

Figure 3D shows liver tissue perfused with antifreeze glycopeptides and frozen with a cooling rate of 4°C/min on a directional solidification stage. The fracture is along the cell membrane, with cells removed in a staggered form leaving behind stair-like arranged hepatocytes 36 (h). The outline of box-like, normal size hepatocytes (h) is shown

by the black arrows 37.

Figure 3E shows normal liver tissue frozen with a cooling rate of approximately 4000°C/min, and fractured along the cell membrane. The cells are removed in a staggered form leaving behind stair-like arranged, normal size, box-like hepatocytes 38 (h), shown by arrows. The bile duct 39 (bd) (cannaculus) has been preserved intact. The scale bar 40 shown Figure 3A to 3E is 10 micrometer.

Figures 4A, 4B and 4C

Figure 4 includes photographs concerned with the cryopreservation of immature pig oocytes.

Fig. 4A shows one pig oocyte in a transparent droplet during cooling to -130° C. The dark circular rim of the droplet is shown.

Fig. 4B shows a pig oocyte after rapid cooling to -130° C in the AVS solution following 44 hours in vitro culture and stained. The cytoplasm is completely degenerated and the oolemma is not intact.

Fig. 4B illustrates the appearance of an oocyte that was not considered viable after rapid cooling in the AVS solution. The photograph shows an oocyte in which the membrane (oolemma) is not intact and no nuclear details are visible.

Fig. 4C shows an oocyte that survived rapid cooling to cryogenic temperatures in an AVS solution with AFGP and consequently underwent in culture nuclear maturation to the MII stage. It must be emphasized that this is the first time any method has been developed under any conditions in which pig oocytes survive and develop in vitro after exposure to cryogenic temperatures.

Fig. 4C shows the appearance of an oocyte that reached the MII stage after rapid cooling to -130° C. The nuclear development stage is evident.

Fig. 4D shows a pig oocyte after rapid cooling to -130° C in the AVS solution with antifreeze glycoproteins like in Table 1 (40g/ml showing a normal morphology but no nuclear maturation (g.v. stage) after 44 hours of incubation: intact oolemma, intact g.v. membrane, normal cyto-

plasm morphology (the bottom of the photograph shows some cummulus cells). The scale bar for Fig. 4 is 50 μm.

Fig. 4D shows an oocyte cooled rapidly in the AVS solution with 40 mg/ml AFGP. This oocyte was not considered viable because it did not undergo nuclear maturation (remained at the g.v. stage). Nevertheless, it is noted that the cells show a normal morphology with an intact oolemma and an intact g.v. membrane. The intact appearance of the oolemma in Fig. 4D is typical to all oocytes cooled in the presence of the AFGP solution without AFGP, (Fig. 4B) for an illustration of the effect of the AFGP. This set of experiments clearly demonstrates that the AFGP has a cryoprotective effect, which is associated with retaining the integrity of the cell membrane when exposed to severe temperature conditions.

Figures 5A and 5B

Fig 5 includes photographs concerned with cryopreservation of pig embryos at the two cell stage. The scale bar for Fig. 5 is 50 μm.

Fig 5A shows a two cell embryo after rapid cooling to -130° C in the AVS solution and 24 in incubation. The complete disintegration of the membrane is evident.

Fig. 5A shows the appearance of a pig embryo that was cultured after rapid cooling in the AVS solution. The cell is obviously not viable and the disintegration of the blastomere membrane is complete.

Fig 5B shows a normal four-cell stage embryo developed from a two-cell stage embryo after rapid cooling to -130°C. The upper right-hand side of the photograph shows an embryo that remained at the two-cell stage. The clear integrity of the membrane is evident even in embryos that failed to develop. The scale bar for Fig. 5 is 50 μm.

Fig. 5B shows a pig embryo at the four-cell stage after rapid cooling in the AVS solution with AFGP and after in vitro culture. Obviously the embryo shown in Fig. 5B survived the exposure to cryogenic temperatures and developed in a normal way, in vitro. Again, this is the first report of a method for successful cryopreservation of pig

embryos at the two-cell state.

Additional important information can be seen in Fig. 5B. The upper right hand side of the photograph shows a pig embryo that remained at the two-cell stage, i.e., it did not survive rapid cooling according to our criteria. Nevertheless, the clear integrity of the blastomere membrane is noted and compared to the appearance of the disintegrated membrane in Fig. 5A.

Figure 6A, 6B and 6C

Fig. 6 includes photographs concerned with cryopreservation of mouse embryos at the two-cell stage. The scale bar for Fig. 6 is 50 μm.

Fig. 6A shows one mouse embryo in a transparent droplet during cooling to -130° C in the AVS solution after 72 hours incubation.

Fig. 6B shows one of the embryos remained at the two-cell stage with shrunken blastomere which implies membrane damage. In the second embryo, the blastomere membranes have disintegrated completely;

Fig. 6B illustrates the appearance of mouse embryos that did not survive rapid cooling in the AVS solution. The blastomere in one of the embryos in Fig. 6B are shrunk which implies loss of membrane integrity. The membrane disintegration is nearly complete in the blastomere of the other embryo shown in the photograph. The survival of rapidly cooled oocytes and embryos did not improve when 50 mg/ml AP were added to the basic AVS solution. The cell membrane integrity did not improve either and, in fact, there was absolutely no difference in survival or morphology between embryos and oocytes cooled in the AVS solution or the AVS solution with AP.

Fig. 6C shows the typical appearance of a blastocyst following in vitro culture of a two-cell stage mouse embryo cooled to -130° C in the presence of the AFGP.

The typical appearance of normal mouse blastocysts following cooling in the AVS solution with 40 mg/ml AFGP is shown in Fig. 6C. The high rate of survival of mouse embryos in the presence of AFGP's, 82.5% compared to no

survival, 0% without AFGP's, provides a clear indication of the cryoprotective properties of the AFGP.

As significant as these results are on survival, equally important are the microscopic observations showing that the integrity of the oolemma in the pig oocytes and of the blastomere membrane in the pig embryos is retained when the cooling occurs in the presence of AFGP. The microscopic evidence shows that the membrane was intact in 35 of the 45 pig oocytes (82.2%) and in the blastomere of 23 of the 23 (100%) pig embryos when the oocytes and embryos were cooled rapidly to cryogenic temperatures in the presence of AFGP. The integrity of the two-cell stage pig embryo was discussed earlier with respect to Fig. 5B.

The results of Figures 5 and 6 show that the addition of 40 mg/ml of AFGP dramatically improved the survival of the embryos and the oocytes, with 24.5% and 26% survival for the pig oocytes and pig embryos, respectively and 82.5% survival for the mouse embryos.

Because the mouse embryo provides such an unambiguous criteria for viability and because the survival in the presence of AFGP is so high, this animal model was particularly useful for the parametric studies. The results of the parametric studies on the effect of AFGP concentration are also listed in Table 3 above, and show a sudden transition between very high survival at concentrations higher than 20 mg/ml AFGP to very low, or no survival at concentrations lower than 10 mg/ml.

The results presented here clearly demonstrate that the AFGP's facilitate the survival of different animal models at cryogenic temperatures. The results also show that the mechanism of protection is associated with the ability of the AFGP's to maintain the integrity of the cell membrane during exposure to cryogenic temperatures. AFGP's compounds that modify the process of freezing in solutions in a similar form to the AFGP's, sometimes have no effect on maintaining the structural integrity of the membrane.

Figures 7A, 7B and 7C

Figures 7A, 7B and 7C show pig oocytes preserved for

4hr at 4°C without AFGP (7A), and with 40m/ml AFGP (7B and 7C). These figures show that the oolema is damaged without the AFGP(7A). It stays intact with AFGP even in cells that do not survive, and it also facilitates the in vitro development of ooytes to the MII stage Figure (7C). The results in Table 4 suggest that the addition of antifreeze glycoproteins is useful in protecting cell membranes and in blocking ion channels. The observation that antifreeze glycoproteins 1-5 and 7-8 (Table 4, Fractions) separately do not protect the ion flow as well as the 1-5 and 7-8 together suggests that each one of the proteins is active in protecting different proteins and ion channels, i.e., is specific. Therefore it appears that all of the AFGP's are needed for complete protection, while individually, they offer partial protection.

Figures 8 and 9

Figure 8 is a photogrpah of rat liver perfused with Krebs solution only and cooled to -35°C.

Figure 9 is a photograph of rat liver perfused with a identical Krebs solution for Fig. 8 with 20 mg/ml of AFGP fractions 1-8 as obtained fromt he Antarctic fish (see Table 1).

While not wanting to be bound by theory, the protective effect of the AFGP is probably associated with the particular chemical structure of the molecule. It is possible that the protection afforded to the cell membranes during exposure to cryogenic temperatures is a consequence of bonds formed between the hydrophilic parts of the membrane proteins and the AFGP's. There is evidence that the protection afforded by the AFGP's is concentration-dependent in a nonlinear fashion, which suggests that for complete protection all the bonds must be established and no survival is possible with partial interaction between the cell membrane and the AFGP.

The following examples are presented to further explain, describe and define the present invention. They are not to be construed to be limiting in any manner.

GENERAL EXPERIMENTAL

The phosphate buffer solution (PBS) standard solution and may be supplemented as indicated herein, e.g. the higher molecular weight AFGPs 1 to 5 seems to be strongly related to the modification of the ice crystal structure, the biological function of the low molecular weight AFGPs remains unclear. They are less efficient in depressing the freezing point than the larger glycopeptides yet they seem to be present in the serum at much higher concentrations.

AFGP used from Fractions 1-8 (Table 1) are in essentially the same ratio to each other as is found in the Anarctic fish. Fraction 6 is present in a trace amount in the fish, and its presence or absence in the following experiments (in the concentration of Fraction 1-8) is assumed to have a negligible effect on the experiment.

A preferred concentration of AFP or AFGP in aqueous solution in this invention ranges between about 1 and 50 mg/ml., especially Fraction 1-8, Table 1. For some applications, a range of 20-40 mg/ml is preferred.

As can be observed, ice crystal formation has caused major disruption of the blood vessels and the surrounding cell tissue and cell membrane.

As can be seen the cell membrane structure shows minimum disruption. The cell tissue appear to have remained discrete, the cell membrane appear essentially intact and the blood vessels are not significantly enlarged.

EXAMPLE 1

FREEZING OF LIVER TISSUE

(a) Adult female Sprague-Dawley rats, ages 45 to 50 days were anesthetized with ether throughout the surgical procedure. The abdomen was exposed via a midline incision to expose the liver. The portal vein was exposed and cannulated. Immediately, one thousand units of heparin were injected into the vein. This procedure was followed by the injection of a 5-ml solution of physiological saline containing 200 mg of AFGPs from an Antarctic fish (Dissostichus Nawsoni) (see Table 1), in a physiological composi-

tion of Fractions 1-5 and 7,8 (25/75). Optionally, glycerol/saline is perfused through the liver. The AFPs used are those shown in Table 1 above. The AFPs for Dissostichus mawsoni and for the AFGPs of Table 1 have essentially the same molecular weights and ratios to one another. A combination of antifreeze glycopeptide No. 1-5 and No. 6-8 are used in a ratio of 1/3, w/w. The portal vein was immediately clamped to prevent back flow. Within a period of 2 minutes, several rectangular samples of the liver, 8 by 4 by 3 mm in size, were sectioned with single radial razor cuts approximately 3 mm from the periphery of the lobe and were placed lengthwise on two No. 1 coverslips. A total of four animal experiments were performed.

The first coverslip was immediately plunged into nitrogen slush maintained under vacuum at -213°C. No boiling was visible. The cooling rate during freezing was estimated at about 4000° C/min. At the same time, the other coverslip was transferred to the directional solidification stage described earlier. The samples were frozen from an initial temperature of 25° C to a final temperature of -35°C, with a cooling rate of 4°C/min. The time of freezing was approximately 15 min. After freezing, the frozen samples were immediately immersed in the liquid nitrogen slush and transferred to an AMRAY 1000 low temperature scanning electron microscope (LTSEM). The samples were fractured in the cryochamber of the microscope, exposing an area approximately 2 mm from the outer surface of the lobe, gold-coated and transferred in a frozen hydrated state to the refrigerated stage of the LTSEM.

Photographs obtained from the LTSEM are two-dimensional images of an irregularly fractured three-dimensional surface. The photographs are taken at magnifications varying from 200 to 5000 times.

Figures 2A, 2B and 2C show results from liver tissue perfused with AFGPs and frozen in nitrogen slush. These photographs demonstrate that the AFGPs modify the freezing pattern in mammalian tissue. Figures 2A and 2B illustrate the frozen tissue photographed at a magnification of 1000

times. Figures 2A and 2B were obtained after slight radiant etching of the frozen tissue, show the outline of ice crystals. Shown are individual cells and, in several of the cells, the nucleus is also visible. The ice crystals in the cells are different from those in the blood vessels. The ice crystals inside the cells are similar to typical ice crystals formed from plunging tissue in liquid nitrogen slush. These ice crystals are round in shape with dimensions in the micron range and are uniformly distributed throughout the cells. However, the ice crystals in the blood vessels perfused with AFPs are markedly different. The ice crystal structure is spicular with dimensions in the submicron range. It is also very similar to that observed during freezing of aqueous solutions of AFPs. See Figure 1C. Figures 2A, 2B, and 2C show spicular ice crystals in all the longitudinally and transversely fractured blood vessels and that the spicular ice crystals are oriented in the same direction independent of the relative orientation of the blood vessel.

These results demonstrate that water present in the tissue in the presence of AFGPs have ice crystals which do not propagate in the direction of the blood vessels but, rather, grow with a stable c-axis orientation, presumably in the direction of the temperature gradient. This is consistent with earlier reported research, which show that during freezing in a solution of AFGPs, the solutes entrapped between the spicules, stabilize and force the ice crystal to grow only in the direction of the c-axis. Therefore, the crystal growth is different from the freezing of solutions without AFGPs, where the ice crystal can grow along the different orientations of the ice crystal hexagonal prism facets, allowing a change of direction whenever the ice crystal encounters obstacles, such as the cell boundary. In the presence of the AFPs, the growth in the direction of the c-axis is extremely stable and the orientation of the ice crystal cannot change when the ice crystal encounters a cell boundary. All the spicular ice crystals terminate at the blood vessel boundaries. The ice

crystals in the blood vessels also do not cause the nucleation of the water in adjacent cells.

A higher magnification of a micrograph of the spicular ice crystals in a larger blood vessel is illustrated in Figure 2C. The significant difference between the spicular submicron size of the ice crystals in the blood vessel and the rounded micron size ice crystals in the adjacent cells in evident. The small dimensions of the spicular ice crystals suggest another potential application of the AFPs. Currently, extremely high cooling rates (e.g. 40,000 to 100,000°C/min, several orders of magnitude higher than the cooling rates in the present invention, are used for preparation of tissue samples with very small ice crystals for microscopy. Freezing tissues perfused with AFPs of the present invention is used to produce small ice crystals in tissue with much lower cooling rates which can be easier to achieve experimentally.

Figures 3A, 3B, 3C, 3D and 3E demonstrate the effect of the AFPs on the freezing pattern of mammalian tissue frozen with low cooling rates. The structure of liver tissue frozen with a cooling rate of 4°C/min in the presence of AFPs is illustrated in Figures 3B, 3C, and 3D. These Figures are compared with Figure 3A which shows the structure of liver tissue frozen with a cooling rate of 4°C/min without AFPs and with Figure 3E which shows the structure of liver tissue frozen without AFPs with a cooling rate of approximately 4000°C/min.

(b) For comparison purposes Figure 3A shows the typical structure of liver tissue frozen at low cooling rates without AFPs. The large continuous ice crystals along the sinusoids an the completely dehydrated hepatocytes surrounding the blood vessels are evident. Because of the dehydration of the hepatocytes in liver tissue frozen with low cooling rates, the tissue is not able to fracture along the cell membrane boundaries and, therefore, shows fractures through large ice crystals.

(c) The morphology of liver tissue frozen with low cooling rates in the presence of AFPs is markedly differ-

ent. Figures 3B and 3C show cross-sections through a large blood vessel and the adjacent tissue, at a magnification of 1000x and 2000x, respectively. The submicron size spicular ice structures, typically found in the freezing solutions with AFPs, (Figure 1C) are evident in the blood vessel. All the ice crystals in the blood vessel have the same orientation and they terminate at the blood vessel boundary. The structure of the spicular ice crystals in Figures 3B and 3C are markedly different from the smooth single ice crystal structures observed in the blood vessels of tissue frozen with the same cooling rate but without AFPs, Figure 3A. Figures 2A, 2B, 2C, and 3A, 3B, 3C, 3D and 3E obtained for cooling rates of 4000°C/min and 4°C/min, respectively, demonstrate that the AFPs generate a similar, submicron size stable spicular ice crystal structure when freezing mammalian tissue over a large range of cooling rates.

(d) The fracture in Figures 3B, 3C, 3D and 3E is along the cell membrane, where the cells are removed in a staggered form, leaving behind stair-like arranged hepatocytes. The micrographs show contours of box-like shaped hepatocytes, which do not appear dehydrated and actually retain their normal shape. Comparing Figures 3B, 3C and 3D with Figures 3A and 3E, it is found that the figures possess much more resemblance to Figure 3E, showing identifiable box-like hepatocytes with typical dimensions, fractured at the cell membrane. Figure 3A which shows dehydrated hepatocytes and ice crystals in the expanded sinusoids, is significantly different. The surprising result is that while Figures 3B, 3C and 3D are photographs taken from samples perfused with AFPs and frozen with a cooling rate of 4°C/min, which is similar to the cooling rate used for Figure 3A, Figure 3E is a photograph of liver tissue frozen, without AFPs, in liquid nitrogen slush with a cooling rate of approximately 4000°C/min. As expected, freezing at these high cooling rates retains the normal structure of the hepatocytes, and shows the bile duct with bile along the cell membrane of Figure 3E. For freezing with low cooling rates (4°C/min) without AFPs, it is

impossible to fracture the dehydrated hepatocytes along the cell membrane, and these micrographs always show ice crystals. The observation is that the structure of liver tissue frozen with 4°C/min in the presence of AFPs resembles that of tissue frozen with a cooling rate of 4000°C/min. This result illustrates the significant effect of the AFPs on the freezing pattern in mammalian tissue.

EXAMPLE 2

CRYOPRESERVATION OF WHOLE ORGAN

(a) Cryopreservation of a whole liver from a rat as described in Example 1 is adapted for a whole organ. The rat liver is surgically removed, and held in an aqueous solution at 24°C. The portal vein vessel is cannulated. The well known Langendorf perfusion system (with a first bottle containing Krebs solution) is used. See procedure, for example, D.E. Pegg et al. (1986), Cryobiology, Vol. 23, pp. 150-160.

A second bottle of solution contains a saline solution and appropriate quantities of glycerol, dimethyl sulfoxide, ethylene glycol, polyvinyl chloride glucose or mixtures of these substances which are known as protectants for cells together with antifreeze glycopeptides at a concentrations of 40 mg/ml.

A computer controlled mixing switch provides intermediate amounts of solution 1 (Krebs solution), solution 2 (glycerol/saline/AFGP solution) bursts of time lengths controlled by the computer, for example 0.01 sec. to 0.1 sec. to 1 sec. time lengths. The two solutions mix in a specially provided mixing chamber.

These two bottles of physiological solution are connected to a mixing valve having known adjustable flow rates (e.g. about 5 ml/sec) and a computer to accurately vary the flow rate and mixing of each bottle's contents immediately prior to perfusion. The perfusion using the solutions of bottles 1 and 2 is well known in the art as described by G. N. Alink et al. (1976), Cryobiology, Vol. 13, pp. 295-304; (1977) Cryobiology, Vol. 14, pp. 409-417 and 399-408; and (1978) Cryobiology, Vol. 15, pp. 44-58,

and K.E.F. Hobbs et al. (1969), Cryobiology, Vol. 6, pp. 239-245. The Krebs solution is perfused through the liver held at 24°C at a rate of 4 ml/min.

The glycerol/saline/AFGP concentration in the perfusate is slowly increased at a rate of 0.001-mole/0.1 sec. until a concentration of about 3 mol glycerol and 40m/ml AFGP is perfused. The tissue is then perfused with the 3 mol glycerol/saline for an additional 20 min. The perfused liver in solution (AFGPs 0.001 M in the organ) is next placed in a cooling stage (US Patent - 4,531,373) and the temperature of the whole perfused liver is then cooled at a rate of 1°C per minute until -150°C is achieved. The liver is then cooled using a liquid nitrogen slush to 196°C and held at this temperature for 72 hrs. The frozen liver is then carefully thawed at a rate of between about 0.1 to 10°C per min. (preferably about 1°C per min.) using known techniques with warm fluids. Alternatively, carefully controlled microwave heating is used to thaw the perfused liver. When the thawed liver is about 0°C, a nutrient solution of Krebs is perfused through the large cannulated blood vessel. When warmed to 37°C, the thawed liver recovers not only tissue function but also organ function. The viability of the organ is measured by the production of bile following the freezing and careful thawing.

(b) When the rat liver in subpart (a) above is replaced with a rat kidney and the procedure is repeated, a thawed kidney having viable tissue function and recovered organ function is obtained.

(c) When the rat liver of subpart (a) is replaced with a rat heart, some additional procedures particular to heart tissue for perfusion, including immediate removal of blood from the heart chambers, are observed. After freezing of the antifreeze polypeptide perfused heart, careful thawing and perfusion with appropriate biological fluids, the reactivated heart having viable tissue function and viable organ function is obtained. The viability of the

heart is measured by observing restored contractions of the heart muscle.

EXAMPLE 3

CRYOPRESERVATION OF WHOLE MAMMAL

(a) The cryopreservation of a whole rat is adapted from Examples 1 and 2. An adult rat is anesthetized and the carotid vein is cannulated.

A computer controlled mixing switch provides intermediate amounts of solution 1 (Krebs solution), solution 2 (glycerol/saline/AFGP solution) bursts of time lengths controlled by the computer, for example 0.01 sec. to 0.1 sec. to 1 sec. time lengths. The two or three solutions are smoothly mixed in the delivery tube or optionally in a specially provided mixing chamber.

The Krebs solution is first perfused briefly through the animal. Heparin 2,000 units is added to inhibit blood clotting.

A solution known as a cryopreservative for cells, such as 3 molar glycerol in saline with 40 mg/ml AFGP, is perfused through the organ using the mixing switch arrangement described herein. Optionally, blood substitutes such as saline and/or known fluorinated hydrocarbons are added to the perfusion solution.

The rat is then perfused with a glycerol/saline/AFGP solution (of Example 1) at a pulsed rate of 10 ml/min for 20 min. The body temperature of the rat is lowered to less than 2°C. The animal is then cooled from the exterior to the interior at a rate of 1°C/min.

The rat is then cooled to -150°C and held at this temperature for 7 days. The frozen animal is then carefully thawed at a rate of 1°C/min to 0°C in the presence of oxygen/nitrogen (20/80, v/v). A nutrient blood substitute solution such as Krebs, Euro Collins, UW solution containing is perfused through the rat via the carotid vein, the frozen animal is warmed slowly to its normal biological temperature (about 37°C). Tissue function, organ function and viable whole rat function is recovered.

EXAMPLE 4

CRYOPROTECTION OF IMMATURE PIG OOCYTES AND PIG EMBRYOS

Immature pig oocytes were isolated from selected follicles of cyclic sows 20 minutes after slaughter at 20° C, according to the procedure by Mattioli, et al. (20). The two-cell stage pig embryos were collected from prepubertal gilts (average weight 90 kg.). Estrus induction was carried out by administration of 1250 I.U., Pregnant Mare Serum Gonadopin (PMSG), (SIGMA, St. Louis, Missouri), followed 56 hours later by administration of 750 I.U., Human Chorionic Gonadropin (HCG), SIGMA, St. Louis, Missouri). Two artificial inseminations were performed after 34 hours and 46 hours from the HCG injection. The two-cell embryos were collected from the animal by mid-ventral laparoscopy under general anesthesia 60 hours after the HCG injection.

In preparation for low temperature exposure, the embryos and the oocytes were first introduced in one ml of PBS containing 0.1 M sucrose and 20% FCS at 22° C. This was followed by a three-minute gradual mixing with one ml PBS containing 5% glycerol, 0.1 M sucrose and 35% propylene glycol, according to a procedure developed by Arav (21). The embryos and oocytes were transferred to slides containing 0.1 μl droplets of either AVS or AVS with AFGP or AFP, one embryo or oocyte per droplet. (The experimental conditions and parameters are summarized in Table 4.) Prior to cooling, the pig oocytes and pig embryos were incubated on the slide for 6 minutes at 22° C.

Droplets containing oocytes and embryos were exposed in separate experiments, to the cooling/warming protocol described earlier. The cooling/warming process was monitored using a recording video camera attached to a Leitz Diaplan microscope with magnification of 120x and 340x. Fig. 4A illustrates the typical appearance of pig oocytes inside transparent droplets, during cooling with a rate of 1700° C/min to -130° C. In all experiments, the droplets remained transparent at magnification of 340x indicating the absence of visible ice crystals. During warming at

1,700° C/min, the transparent droplets retained an appearance identical to that in Figs. 4A and 6A. The microscopical evidence shows that the morphology of the embryos and oocytes did not change during cooling and warming.

After warming, in preparation for viability assays in cell culture, the pig oocytes and pig embryos were introduced for three minutes in 1 ml PBS, with 20% FCS and 1 M sucrose at room temperature (22° C), followed by transfer to and equilibration in PBS containing 20% FCS for 10 minutes at 22° C.

Prior to cell culture, all embryos and oocytes were washed three times in cell culture media. The pig oocytes were cultured in TCM-199 medium which was modified in that it contained 5 μg/ml of sheep luteinizing hormone (NIH S20), pig follicle stimulating hormone (LER 441-2) and 20 ng/ml of pig prolactin (LER 2073). The pig embryos were cultured in Brinster culture medium without glucose. After equilibration in cell culture medium, the oocytes and the embryos were incubated at 37° C under 5% CO_2 in air, the pig oocytes for 44 hours, and the pig embryos for 24 hours.

The pig oocytes were fixed after 44 hours incubation in acetic alcohol (1:3 v/v) and stained with lacmoid stain. The viability of immature pig oocytes was assessed using phase contrast microscopy (20), by their ability to develop from the germinal vesicular, (g.v.) stage to the first metaphase (MI) or second metaphase (MII) stage in vitro, and to present a normal morphology (cytoplasmatic compactness, integrated oolemma, visible nuclear stage). The viability of the two-cell stage pig embryos was assessed by their ability to develop to the four-cell stage in culture, while maintaining integrated morphology (cell membrane and cytoplasm). The <u>in vitro</u> culture was stopped at the four-cell stage because many times early stage pig embryos encounter the four-cell block when cultured in vitro (22), and therefore, further incubation would not be useful to an experimental goal to assess the viability of the embryos after exposure to cryogenic temperatures.

EXAMPLE 5

CRYOPROTECTION OF MOUSE EMBRYOS

The procedure of Example 4 was followed except for the following changes.

Mouse embryos at the two-cell stage, were obtained from four-week old $C_{57}Bl/GJ$ mice which were paired singly with CBA/CaJ males. The females were induced to superovulate by intraperitoneal injection of 5-7.5 I.U. PMSG (SIGMA, St. Louis, MO) followed 48 hours later by 5-7.5 I.U. HCG (SIGMA, St. Louis, MO). Forty hours after insemination, the oviducts were excised from the mice and the two-cell embryos flushed out and stored in phosphate buffeted saline, (PBS) medium.

In preparation for low temperature exposure, the mouse embryos were introduced in 1 ml of PBS and FCS as described in Example 3. (The experimental conditions are also summarized in Table 7.) Prior to cooling, the mouse embryos were incubated on a slide for 12 min. at 4° C.

Fig. 6A illustrates the typical appearance of mouse embryos inside transparent droplets during cooling with a rate of 1,700° C/min to -130° C. In all experiments the droplets remained transparent at a magnification of 340x indicating the absence of visible ice crystals. During warming with 1700° C/min. the transparent droplets retained an appearance identical to that in Figs. 4A and 6A. The microscopical evidence shows that the morphology of the embryos and oocytes did not change during cooling and warming.

After warming, in preparation for viability assays in cell culture, as described in Example 3, the mouse embryos were exposed for three minutes at 4° C to 1 ml of PBS, with 20% FCS and 1 M sucrose, followed by transfer to and equilibration in PBS containing 20% FCS for 12 minutes at room temperature (22° C) (21). Prior to cell culture, all embryos were washed three times in cell culture media. The mouse embryos were cultured to T_6 Whittingham medium (21).

After equilibration in cell culture medium, the mouse embryos were incubated at 37° C under 5% CO_2 in air for 72

hours.

The viability of the mouse embryos after exposure to cryogenic temperatures was assessed by their ability to develop in vitro to the blastocyst stage while showing normal expanded morphology. Table 1 lists the experimental results which were discussed earlier.

EXAMPLE 6

CELL MEMBRANE ELECTRICAL POTENTIAL

During the experiments in which pig oocytes were vitrified in the presence of AFGP's, it was discovered that only 24% of the oocytes and 26% of the pig embryos survived the rapid cooling. However, it was surprisingly observed that close to 100% of the cell membranes remained intact. On the other hand, without the AFGP's (100%) of the cell membranes were destroyed. The effect of the AFGP's at temperatures higher than 0° C for pig oocytes was examined because it is reported that they could not survive at temperatures lower than +10° C.

Immature pig oocytes were obtained from selected follicles of cylcic sows 20 minutes after slaughter, at 20°C, according to the procedure by Mattioli et al. The oocytes were then introduced in vials containing different concentrations of AFGP (Fractions 1-8) in a saline supplemented with 0.4 w/v microm/ml BSA (Bovine Serum Albumin), 0.34 mM pyruvate, 5.5 mM glucose and 70 micromol/ml kanamycin). The AFGP's used in this work were obtained from Antartic fish belonging to the family Notoheniidae (Dissostichus Nawsoni). A physiological composition of AFGP's was used in most of the experiments having one part by weight of AFGP's 1 to 5, and three parts by weight of AFGP's fractions 7 and 8. (available form A.L. DeVries supra.) Experiments were also performed with AFGP's fractions 1-5 and AFGP's 7 and 8, separately. The different experimental parameters are listed in Table 1. To determine the protective effect of the AFGP the oocytes were exposed for 4hr and 24hr to a constant temperature of 4°C, in a constant temperature chamber. After removing the oocytes from the 4°C environment the integrity of the oolema was determined

by measuring the resting membrane potential of the oocytes at room temperature, 22°C, according to a procedure by Mattioli et al. Intracellular voltage measurements were made using single microelectrodes made from borosilicate glass tubes (Hilgenberg, FDR). The electrodes were pulled on a horizontal puller and filled with 2M KCl. The resistance of the electrodes was 10-20 Megaohms (Mo). To record the membrane potential the tip of the microelectrode was maneuvered to the surface of the cell using a micromanipulator controlled through 400x magnification with a Leitz Fluovert microscope equipped with Nomarski optics. When the tip just dimpled the surface of the cell, the final penetration was achieved by briefly causing an electrical oscillation induced by turning the capacity compensation of the amplifier. The electrical potential values, which remained constant for at least 1-2 sec, were recorded. The resting membrane potential is a very sensitive criteria for membrane integrity. In addition, experiments were performed to determine the viability of certain oocytes following exposure to the hypothermic conditions. Several of the oocytes exposed for 4 hr to 4°C, with 40mg/ml AFGP 1-8 in the basic PBS solution and without, were incubated in TCM-199 medium, which was modified in that it contained 5 microg/ml sheep luteinizing hormone (NIH S20), pig follicle stimulating hormone (LER 441-2) and 20 nanog/ml of pig prolactin (LER 2073) at 37°C under 5% carbondioxide for 44 hr. After incubation the oocytes were fixed in acetic acid/ethyl alcohol (1:2 v/v) and stained by lacmoid stain. The viability of the immature pig oocytes was assessed using phase contrast microscopy by their ability to develop from the initial germinal vesicular (g.v.) stage to the first or second metaphase, MI or MII in vitro and to present a normal morphology (cytopolasmatic compactness, integrated oolema, visible nuclear stage). The microscopic observation also allows a qualitative evaluation of the structural integrity of the membrane.

For each experiment the results for both criteria are given, as shown in Table 4. The mean of all means was -31

mv, and the mean standard distribution, 4.5 mv. These values are within the normal range of membrane potential for pig oocytes. It should be emphasized that measuring potential is a very sensitive and recognized measure of membrane integrity. It is apparent from Table 4 that the combined AFGP's fractions 1-8 protect the olema gainst damage induced by exposing the oocytes to hypothermic conditions. Since there are no ice crystals present at 4°C the protection must occur through an interaction between the Antarctic fish glycoproteins and the oolema. Therefore, this part of the experiment demonstrates that the AFGP's directly protect membranes, which is a property of the AFGP's that has never been reported. The level of protection is not a linear function of the AFGP concentration; it reaches saturation at about 1 mg/ml in the perfusion and drops to low values at 0.1 mg/ml. This is a typical property of protein-protein interactions, which may indicate that the glycoproteins may offer protection by binding to the available sites on the oolema and can provide their protection only if all the sites are occupied. These sites could be the membrane proteins. Table 4 also shows that the whole physiological combination of AFGP's 1 to 8 is needed for protection and that AFGP's 1 to 5 and AFGP's 7, 8 separately do not protect the membrane. This result is extremely surprising because studies on the effects of AFGP's on depressing the phase transition temperature and ice crystal formation show that AFGP's fractions 1-5 depress the phase transition temperature almost as effectively as the whole combination of AFGP's fractions 1 to 8. On the other hand, it is apparent that AFGP's fractions 1 to 5 separately do not protect the cell membrane, and neither do AFGP's fractions 7,8 separately. A possible explanation for this phenomena is that all the different proteins with different lengths are needed to bind to all possible sites on the membrane and to block all the possible leaks sites and ions channels.

The microscope evaluations of the oocytes exposed to 4°C for 4 hr and incubated for 44 hr to verify the results

obtained through measurements of membrane resting potential. In the absence of AFGP's only 2 of 20 oocytes retained an integrated oolemma (10%), and none of the oocytes matured in vitro, (0%). Figure 7A illustrates the appearance of an oocyte preserved at 4°C in PBS without AFGP. The oolema is apparently not integrated and the cytoplasm is degenerated. In contrast, in the presence of 40 mg/ml AFGP, 11 of 18 oocytes retained an integrated oolemma, 61% (Figure 7B). This result further demonstrates that the AFGP's protect the oolema of cells exposed to damaging hypothermic conditions and is consistent with the measurements of the resting electrical potential. Close to 25% of the oocytes survived and matured to the MII stage as illustrated by (Figure 7C).

EXAMPLE 7

CRYOPRESERVATION EFFECT OF THE AFGP'S ON RAT LIVER

The procedure is essentially identical to that described in Example 1.

Experiments were performed with adult female Sprague-Dawley rats, ages 45 to 50 days. The rats were anesthetized with ether throughout the surgical procedure. The abdomen was exposed via a midline incision to expose the liver. The bile duct was exposed and cannulated. Bile was collected and used as a criteria for viability. The portal vein was exposed and cannulated. Immediately one thousand units of heparin were injected into the vein. The liver was released and flushed with a basic Krebs solution through the vein. In some of the models this was followed by an injection through the vein of 3 ml solution of Krebbs containing 20 mg/ml AFGP Fractions 1-8 (in a physiological composition found in the fish) (from the Antarctic fish belonging to the family Nototheniid, Dissostichus Mawsoni). The liver was then introduced into a refrigerator at 4° C for 6 hours. After that time, the liver was removed, perfused with a Krebbs solution at body temperature and kept on a plate maintained at body temperature 38°C. The production of bile was measured as a criteria for viability. This test is a well accepted criteria considered to

provide the best overall indication of viability. The results show that with AFGP's, the bile rate of formation was about 85% of the initial level after 6 hours at 4° C. In the absence of the AFGP, bile rate dropped to about 20% of the normal level. Three animal experiments were performed for both the control and the solution with AFGP.

EXAMPLE 7A

EFFECT OF AFGP ON LONG-TERM VIABILITY OF INTACT RAT LIVER TISSUE

Surgical Procedure

Livers from Spague-Dawley rats (35) of ages 50-55 days are surgically removed. The peritoneal cavity is entered under nembutal anesthesia. The bile duct is cannulated with a PE-30 polyethylene catheter, and the bile is collected for 10 min while the surgery proceeds. After partial mobilization of the liver from adjacent tissue, a 16-gauge TEFLON® intravenous catheter is introduced into the portal vein, and 3.0 mL of perfusion buffer containing 1000 units of heparin is rapidly infused using a 3 mL syringe. The inferior vena cava is transected distally, and the portal vein catheter infused with Krebs solution pre-equilibrated to a 95:5 mixture of O_2 and CO_2 at 0°C from the remainder of the surgical procedure. The inferior vena cava is ligated above the renal veins and freed from adjacent retroperitoneal tissues, and a PE-205 polyethylene catheter is secured in the superior vena cava through an incision in the right atrium. Samples of the effluent are collected at this time. The entire liver is then carefully cut free from the surrounding tissue and washed with warm saline.

Storage and Isolated Organ Perfusion

For the test livers, the perfusion line of TEFLON® is removed and a 3 mL solution of Krebs solution containing 20 mg/mL of AFGP fraction 1-8 from Antarctic notothenoiidae fish (D. Mawsoni) is injected into the catheter. The whole liver is then immediately placed into a container containing cold Krebs solution and is returned to the constant temperature apparatus. The apparatus and liver are kept at

a constant 4°C. The liver is stored for periods of 6, 12 and 24 hr.

After the storage process, the liver is removed and inject with a 20 mL of Krebs solution at ambient temperature to remove the AFGP solution. The liver is immediately inserted in the single pass Largends rf type perfusion circuit described by containing Krebs solution 37°C pre-equilibrated with a mixture of 95% oxygen and 5% carbon dioxide. The flow rate is then increased from 5 to 25 mL/min with careful attention to the position of the liver and catheter. The liver is perfused for 50 min. The effluent from the liver is collected continuously for the intervals 0-5, 5-10 and 10-25 min. In addition, bile is collected in 15 min intervals.

Control

For a stored liver control study, the liver was injected with a 3 mL of Krebs solution. The procedure then followed the test study conditions described above without AFGP addition. For the warm control liver, the liver was immediately inserted in the single pass perfusion circuit, and the necessary effluent and bile samples was taken.

The collected bile from each liver was measured and tabulated. The collected effluent was tested for activity of lactic dehydrogenase (LDH). Enzymatic assays for LDH is performed using standard colorimetric techniques (Sigma Diagnostics KIT 500. A UV-visible spectrophotometer is utilized.

Experiments were performed with 35 rats. Each experimental point represents between 3 and 5 animal experiments.

Bile flow commenced within 3-5 mins. after the liver was connected to the single pass perfusion system. The bile flow was well maintained during the 50 min perfusion. Since rat livers do not produce bile salt, the production of bile from any excised liver could not be sustained for much longer than about 50 minutes. The production of bile reaches a plateau during the second collection and maintains this level for the duration of the perfusion. Figure 10 is a plot of the bile production from the second collec-

tion versus the storage period 6, 12 and 24 hrs. Figure 10 shows the gradual decrease of bile production as the storage period increases. The solid line column represents the bile production from liver stored with AFGP in Krebs solution. The dashed line column represents livers stored with Krebs solution only. There is a significant increase in bile production from livers stored with the AFGP at all the storage times. Although the bile flow decreased significantly after 24 hr. of storage, the bile production with AFGP showed an improvement over liver stored with Krebs solution only.

In the 24 hr storage experiments with AFGP, the typical LDH activities during the perfusion process is shown by the solid line column in Figure 10. The dotted line column represent the results from the control liver perfusion, and the liver stored in Krebs solution are represented by the dashed line column. For the AFGP-perfused stored liver, the release of the enzymes is at a maximum during the first 5 min of the perfusion, and then decreases at later times to near control levels. The Krebs solution stored liver also reaches a maximum during the first 5 min. of perfusion. However, the decrease in activity level at later times remains significantly higher than control levels.

In utilizing the enzyme colorimetric tests, these results from LDH activity show the membrane protective capability of the AFGP. Since leakage of higher levels of LDH activity is reported to be associated with membrane damage, this test provides an indication of the integrity of the hepato cellular membrane. The results of the LDH tests show a significant decrease in activity for livers stored in AFGP, which indicates lessor damage to the cellular membrane. From these results, AFGP clearly provides cellular membrane protection during the storage process. As indicated by the increase in bile production, the AFGP protection of membrane leads to better preservation capability compared to Krebs solution alone.

EXAMPLE 7B

PRESERVATION OF RABBIT HEART

A preliminary experiment and a control experiment were performed in parallel using adult rabbit heart.

Two white laboratory rabbits (2-3 kg each) were anesthetized. Each heart was surgically removed. The control heart was perfused with Krebs solution for 30 sec. at 5°C and injected with 5 ml of standard Krebs solution (5°C). Each heart was immediately placed in a small test tube containing Krebs solution at 5°C and placed into an ice/water bath at about 0°. The other heart was perfused with Krebs solution for 30 sec. at 5°C. The aorta was injected with a standard Krebs solution (at 5°C) containing 20 mg/ml of AFGP's-Fractions 1-5 and 7-8 (Table 1) (25/75, w,w) purified by standard electrophoreses.

The control heart (without AFGP) was held at 0°C for 4 hr, then connected to a Langendorf perfusion system, and perfused with Krebs solution at 37°C (optionally containing some glucose) for one hr. During this time, the aorta beat weakly (or fluttered). At the end of one hour at 37° (physiological temperature), the aortic pressure was about 27 mm of water, aortic flow was negligible, and the flow rate through the heart was about 2 cc/min. This heart was nonvigorous, beating at about 30 beats per minute. Visually the portions of the heart tissue appeared to be dead or dying.

The experimental heart (with AFGP) was also held at 0°C for 4 hr, then connected to a Langendorf perfusion apparatus and perfused with Krebs solution at 37°C (optionally containing glucose). At the end of one hour at 37°C, the aortic pressure was over 100 mm water, aortic flow was 12 cc/min, and a cardiovascular flow rate of about 47 cc per min. was measured. This heart was vigorous, beating about 160 beats per minute. Visually, the heart looked robust, these measurements were close to the values for a normal heart.

EXAMPLE 8

Mouse embryos at the two cell stage were introduced in

a T6 Whittingham medium with 40 mg/ml antifreeze glycopeptides and without an atmosphere of 5% CO_2 and a temperature of 37°C for 72h. Accidentally the concentration of CO_2 increased to about 8% and the temperature fluctuated while being most of the time at temperatures higher than 40°C. After incubation at these non optimal environmental conditions close to 80% of the mouse embryos developed to the blastocyst stage in the presence of the antifreeze protein while less than 50% developed to the blastocyst stage without the antifreeze protein. This result demonstrates another useful property of the antifreeze proteins at hyperthermic temperatures and in a chemical environment that is not compatible with the cells.

While only a few embodiments of the invention have been shown and described herein, it will become apparent to those skilled in the art that various modifications and changes can be made in the preservation composition and method to preserve viable plant or animal cells, or viable tissue or viable organs or plants viable animals which cells, tissue, organs or plant or animal are viable upon thawing without departing from the spirit and scope of the present invention. All such modifications and changes coming within the scope of the appended claims are intended to be carried out thereby.

WE CLAIM:

1. A composition useful in the protection and preservation of viable plant or animal cell membranes subjected to hypothermal or hyperthermal or nonphysiological chemical conditions, which composition comprises:

at least one biologically compatible substance and

a biologically compatible aqueous solution.

2. The composition of Claim 1 wherein the biologically compatible substance enhances the viability of cells rapidly cooled in vitrifying solutions.

3. The composition of Claim 2, which further comprises glycerol, ethylene glycol, propylene glycol, dimethyl sulfoxide, polyvinylpyrrolidone, glucose, propanediol, carboxymethyl cellulose, or mixtures thereof.

4. The composition of Claim 1 wherein viable animal cell membranes, are selected from ova, sperm, embryos, cells, tissue, whole organs, or a whole animal.

5. The composition of Claim 1 wherein the antifreeze glycopeptide are used in viable preservation of cells, ova, sperm, oocytes, embryos, enzymes, tissue, organs, or whole plants or animals subjected to hypothermal or hyperthermal temperatures ors, nonphysiological chemical conditions regeneration of the viable biological entity; in the medical treatment of tissues injured by thermal, radiation, or chemical conditions; in the preservation of food; in cosmetics used to restore, preserve, or repair skin tissue; and medical treatment of diseases associated with imbalance of the cell sodium-potassium pump.

6. The composition of Claim 1 wherein the biologically compatible substance is selected from peptides substantially identical to antifreeze glycopeptide obtained from the Antarctic nototheniidae fish as fractions 1 - 5, 6, 7 and 8.

7. The composition of Claim 6 wherein, of the antifreeze glycopeptide present, about 25% by weight comprises fractions 1 - 5 and about 75% by weight of fractions 7 and 8.

8. The composition of Claim 1 for use in the preser-

vation of cell membranes in human cells, tissues, or organs or a whole human being.

9. A method of protecting or preserving viable plant or animal cell membranes which method comprises contacting the viable plant or animal cell membrane with an acceptable concentration of the composition of Claim 1.

10. The method of Claim 1 wherein the concentration of the biologically compatible substance is present in between about 20 and 50 mg/ml of solution within the blood vessels.

11. A composition useful in the preservation at high temepratures or at low temperatures below the normal physiological freezing point of animal fluids or in nonphysiological chemical conditions, at least one animal organ, or animal tissue or whole animal, which composition comprises:

at least one biologically compatible substance which promotes ice crystal growth along the c-axis of the ice crystal, and inhibits growth of an ice crystal along the a-axes of the ice crystal, and

a biologically compatible aqueous solution.

12. The composition of Claim 11 wherein the at least one biologically compatible substance comprises a polypeptide, glycopolypeptide, or a polypeptide or glycopeptide covalently bonded to a carrier, or mixtures thereof.

13. The composition of Claim 12 wherein the polypeptide or glycopolypeptide is obtained from a natural animal source or is substantially identical to a polypeptide or glycopolypeptide obtained from a natural animal source.

14. The composition of Claim 13 wherein:

the polypeptide comprises multiple regions of alanine-alanine-threonine- or -alanine-alanine-alanine-, or

the glycopolypeptide which comprises multiple regions of alanine-alanine-threonine, wherein covalently attached to substantially all of the threonine residues is the disaccharide, β-D-galactopyranosyl-(1→3)-2-acetamide-2-deoxy-α-D-galactopyranose.

15. The composition of Claim 14 wherein the molecular

weight of the polypeptide or glycopeptide is between about 2,000 and 50,000 daltons.

16. The composition of Claim 14 which further independently includes compounds independently selected from glycerol, dimethyl sulfoxide, ethylene glycol, poly-vinylpyrrolidone, glucose, propanediol, carboxymethyl cellulose or mixtures of these compounds which are known to protect individual cells from damage by freezing.

17. A composition useful in the preservation at high temperatures or at low temperatures, below the normal physiological freezing point of animal fluids, or in a nonphysiologicla chemical conditions, of animal cells, animal tissue, at least one animal organ or whole animal, which composition comprises:

at least one biologically compatible macromolecule having alternating hydrophobic regions and hydrophilic regions which repeat between about each 16 to 17 Angstroms, or between about each 19 to 20 Angstroms, and

a biologically compatible aqueous solution.

18. The composition of Claim 17 wherein the alternating hydrophobic regions and hydrophilic regions repeat about each 16.5 or about each 19.5 Angstroms.

19. The composition of Claim 17 wherein the macromolecule is obtained from a fish, amphibian, bird, invertabrate, or reptile.

20. The composition of Claim 13 wherein the tissue, organ, or whole animal is from or is a human being.

21. The composition of Claim 20 wherein the biologically compatible macromolecule comprises at least one antifreeze glycopeptide.

22. The composition of Claim 21 wherein the antifreeze protein is selected from peptide, glycopeptide, or peptide covalently bonded to a biologically compatible carrier.

23. The composition of Claim 22 wherein the molecular weight of the antifreeze glyco peptide is between about 2,200 and 40,000 daltons.

24. The composition of Claim 23 wherein the anti-

freeze peptide is similar to, obtained from or derived from the serum or fluids of an animal selected from amphibians, reptiles, insects, worms, Arctic fish, or Antarctic fish.

25. The composition of Claim 22 wherein the biologically compatible carrier is selected from an antibody, gelatin, biocompatible polymer, peptide, sugar or carbohydrate.

26. The composition of Claim 1 which is useful in the survival, functionality, stability and structural integrity of biological materials, including proteins, lipids, enzymes, cell membranes, animal or plant cells, microorganisms, tissues, organs, whole animals, or whole plants subjected to nonphysiological temperatures, either higher or lower than the normal physiological temperatures or nonphysiological chemical environments by interacting with the proteins, lipids or the cell membranes.

27. The composition of Claim 1 which is useful in improving survival, functionality, stability and structural integrity of biological materials, including, proteins, enzymes, lipids, cell membranes, animal or plant cells microorganisms, tissues, organs, whole animals or whole plants subjected to temperatures lower than 0°C in the presence of ice crystals by modification of the structure of the ice crystals in the immediate vicinity of the proteins, enzymes, lipid or cell membranes.

28. The composition of Claim 1 which is useful in improving in improving survival, functionality, stability and structural integrity of biological materials, including, proteins, enzymes, lipids, cell membranes, animal or plant cells microorganisms, tissues, organs, whole animals or whole plants subjected to temperatures lower than 0°C by reducing the number and the size of the ice crystals or by completely eliminating the ice crystals in the immediate vicinity of the proteins, enzymes, lipids or the cell membranes.

29. The composition of Claim 1 which is useful in improving survival, functionality, stability and structural integrity of biological materials, including, proteins,

enzymes, lipids, cell membranes, animal or plant cells microorganisms, tissues, organs, whole animals, or whole plants subjected to temperatures lower than 0°C in the presence of ice crystals by modifying the mode in which solutes are rejected by the ice formation and thereby changing the chemical composition of the solutions surrounding the proteins, enzymes, lipids or cell membranes.

30. The composition of Claim 1 which is useful to block ion channels in membranes and to stabilize cell membranes.

31. The composition of Claim 1 which is useful in binding other macromolecules or conjugates to proteins, enzymes, lipids, or cell membranes.

32. The composition of Claim 1 wherein the biologically compatible substance is a macromolecule obtained from or substantially identical to a macromolecule derived from an animal selected from fish, amphibian, invertebrates or reptile.

33. The composition of Claim 1 wherein the biologically compatible substances are antifreeze protein similar to, obtained from, or derived from the body fluids of animals selected from insects, amphibians, reptiles, worms, fish, from Arctic, Antarctic, North Temperate or South Temperate zone.

34. The composition of Claim 33 wherein the physiologically compatible substance is from the body fluids of Antarctic or Arctic fish.

35. The composition of Claim 34 wherein the biologically compatible substance is an antifreeze glycopeptide substantially identical to or obtained from or derived from the Antarctic fish is selected from the family Nototheniidae, including the species D. Mawsoni and P. Borchgnevinki, or the Antarctic eel pout Rhigophile dearborni, or the Arctic winter flounder.

36. The composition of Claim 1 wherein the biologically acceptable substance is selected from a polypeptide, a glycopeptide, a polypeptide covalently bonded to biologically acceptable carrier, a glycopolypeptide covalently

bonded to a carrier or mixtures thereof.

37. The composition of Claim 1 wherein the biologically compatible substance has the property of promoting ice crystal growth along the c-axis of the ice crystal, and inhibits growth of an ice crystal along the a-axis of the ice crystal.

38. The composition of Claim 1 wherein the at least one biologically compatible macromolecule has alternating hydrophobic regions and hydrophilic regions which repeat between each 16-17 or 19-20 Angstroms, or which repeat between about each 16.5 or 19.5 Angstroms.

39. The composition of Claim 1 further including additional preserving, protecting or vitrifying compounds selected from glycerol, dimethyl sulfoxide, ethylene glycol, polyvinyl pyrrolidone, glucose, sucrose, propanediol, propylene glycol, carboxymethyl cellulose, or mixtures of these agents which are known to protect cells and biological materials against freezing damage or to promote vitrification.

40. The composition of Claim 1 wherein the biologically compatible compounds are selected from particular antifreeze glycoproteins from Antarctic fish, and antifreeze peptide from Arctic fish or antifreeze proteins derived from insects or worms are used in viable preservation and are used for protection of proteins, lipids, enzymes, cell membranes, cells including oocytes, embryos, microorganisms, tissues, organs or whole animals or whole plants subjected to nonphysiological temperatures and chemical environment.

41. The composition of Claim 1 used to protect and stabilize membranes are also used in preservation of food; in cosmetics to restore, preserve or repair skin tissue; or in diseases associated with the instability of cell membranes.

42. The composition of Claim 1 used to block ion channels in treating diseases associated with imbalances of the intracellular-extracellular ion transport.

43. The composition of Claim 1 used to attach and

interact with cell membrane, in attaching various macromolecules or conjugates to the antifreeze proteins and facilitating their attachment to the cell membrane.

44. A method for enhancing the preservation, survival, functionality, stability and structural integrity of biological materials, at nonphysiological temperatures or in nonphysiological chemical compositions, including proteins, enzymes, lipids, cell membranes, animal or plant cells microorganisms, tissues, organs, animals whole or whole plants, which method comprises:

(a) bringing in contact with the biologically acceptable substance of Claim 1 in sufficient concentration to interact with all the proteins, enzymes, lipids, cell membranes, animal or plant cells, microorganisms, tissues, organs, whole animals or whole plants,

(b) exposing the combination of step (a) to the nonphysiological condition, and

(c) removing the macromolecule and returning the proteins, enzymes, lipids, cell membranes, animal or plant cells microorganisms, tissues, organs, whole animals or whole plants, to a physiological temperature and composition.

45. The method of Claim 44 wherein the nonphysiological condition include hypothermal temperatures about 0°C for preservation of proteins, enzymes, lipids, cell membranes, animal or plant cells, microorganisms, tissues, organs, whole animals or whole plants.

46. A method for the preservation of proteins, enzymes, lipids, cell membranes, animal or plant cells, microorganisms, tissues, organs, whole animals or whole plants at temperatures below 0°C to 4K, which method comprises:

(a) bringing in contact with the biologically compatible substance of Claim 1 optionally with addition the other cryoprotective compounds;

(b) cooling to cryogenic temperatures by means cooling and either vitrifying or freezing the system according to the various concentrations and cooling

rates used higher concentrations of additional compounds, such as propylene glycol or glycerol and higher cooling rates which lead to vitrification and to lower freezing temperature.

(c) maintaining the proteins, lipids, cell membranes, animal or plant cells microorganisms, tissues, organs, animals or plants, animal of these temperatures for time periods of up to 10 years,

(d) warming by warming means including warm fluids or microwave heating to physiological temperature conditions, and

(e) removing the antifreeze glycoproteins and the other compounds and replacing them by physiological compatible solutions.

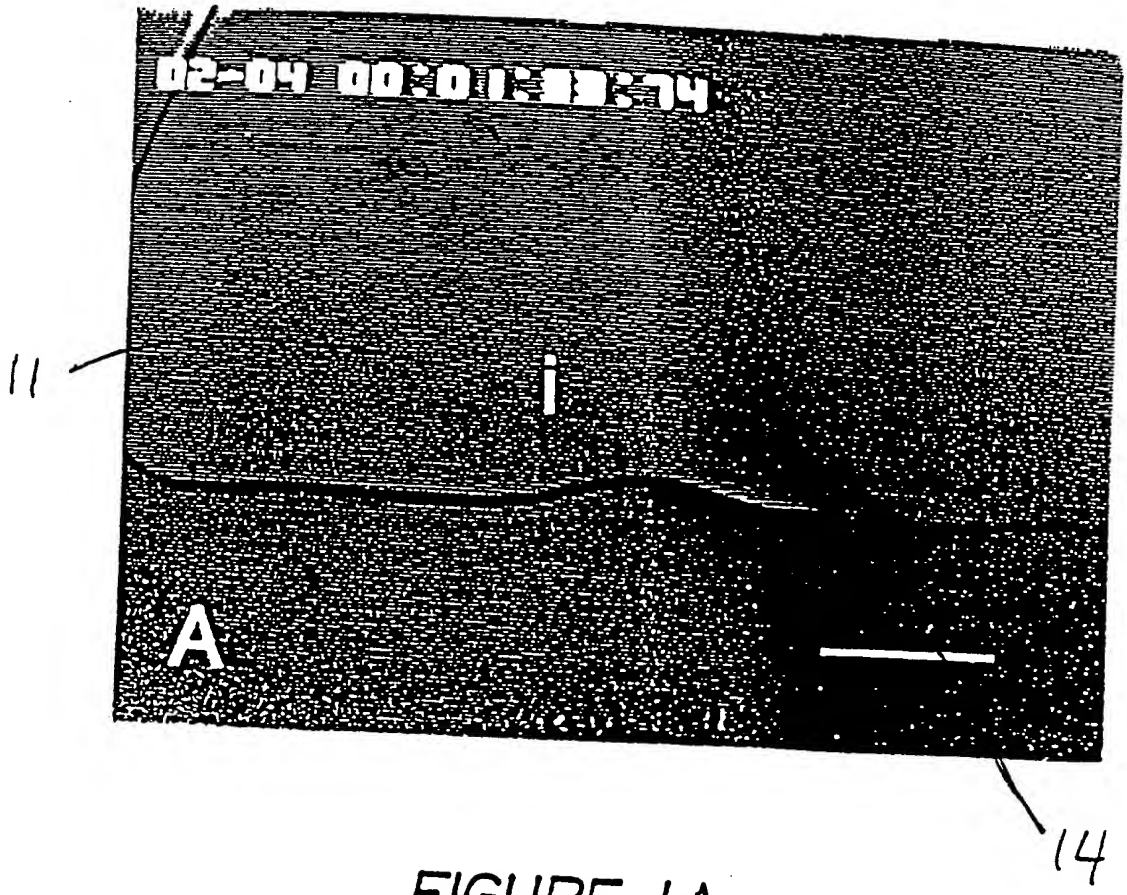


FIGURE 1A

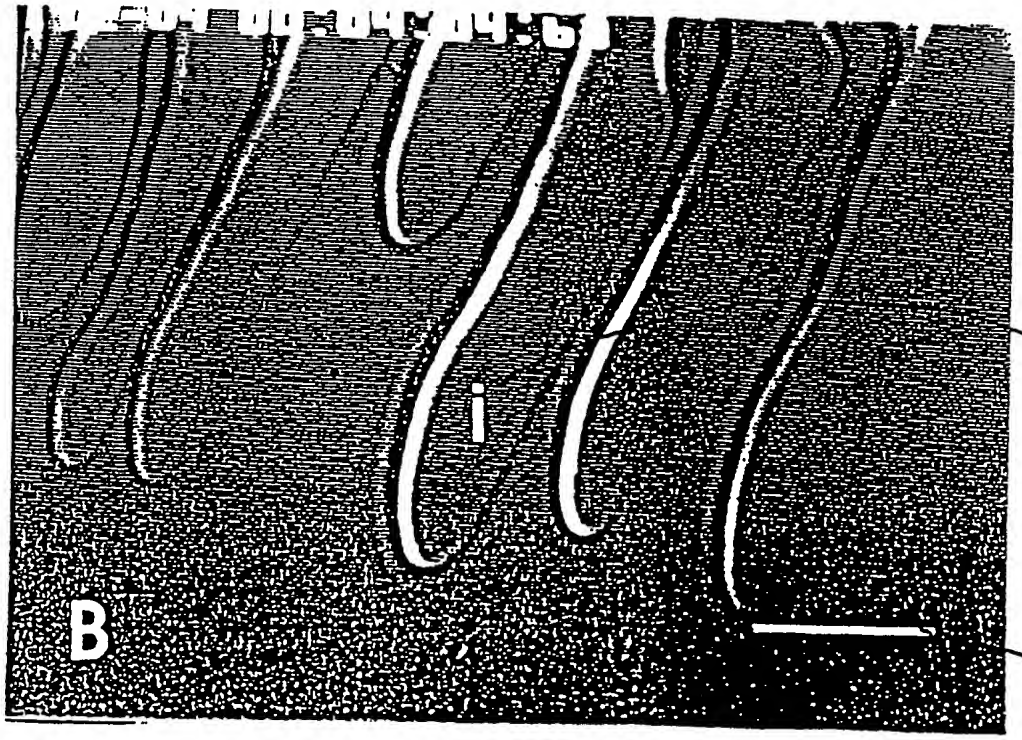
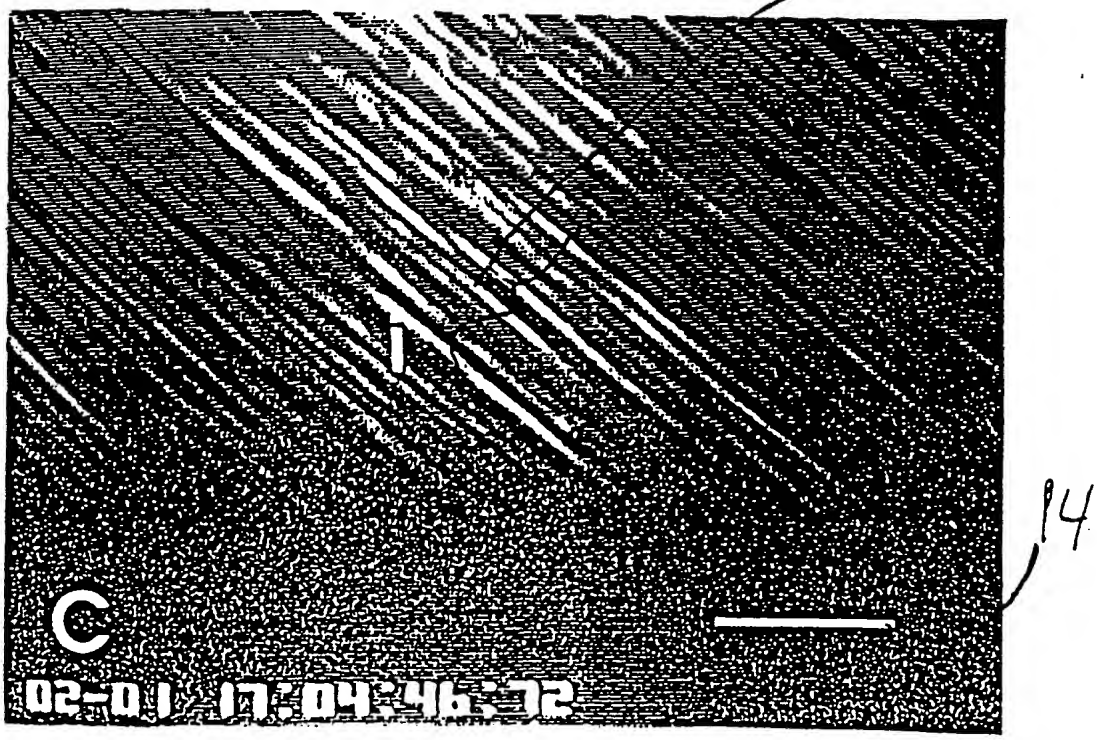


FIGURE 1B

FIGURE 1C



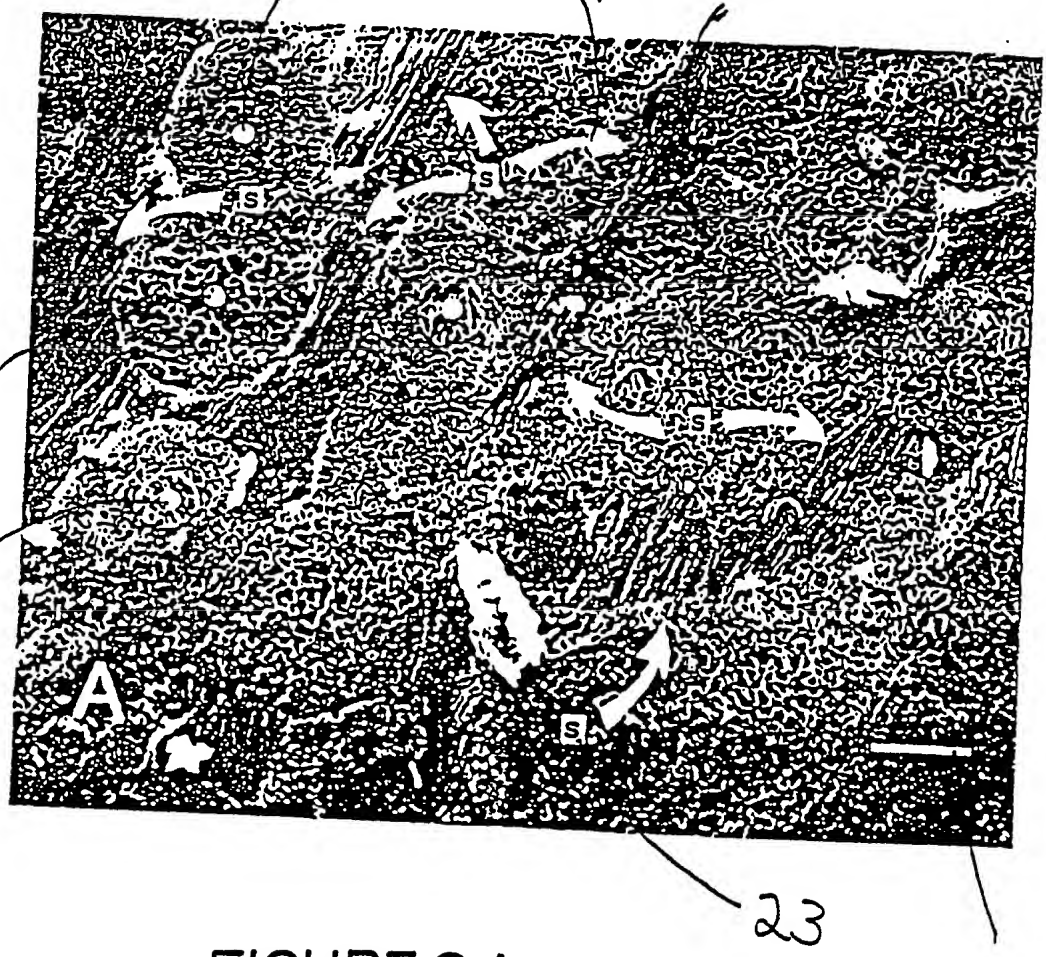


FIGURE 2 A

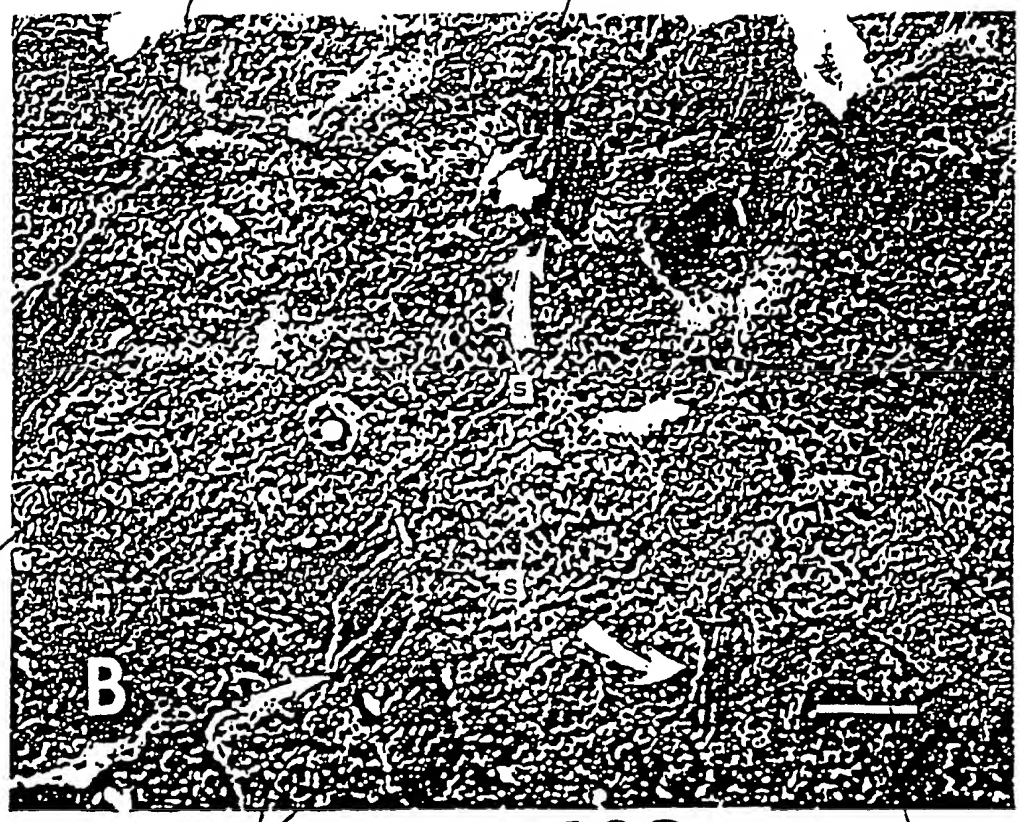


FIGURE 2 B

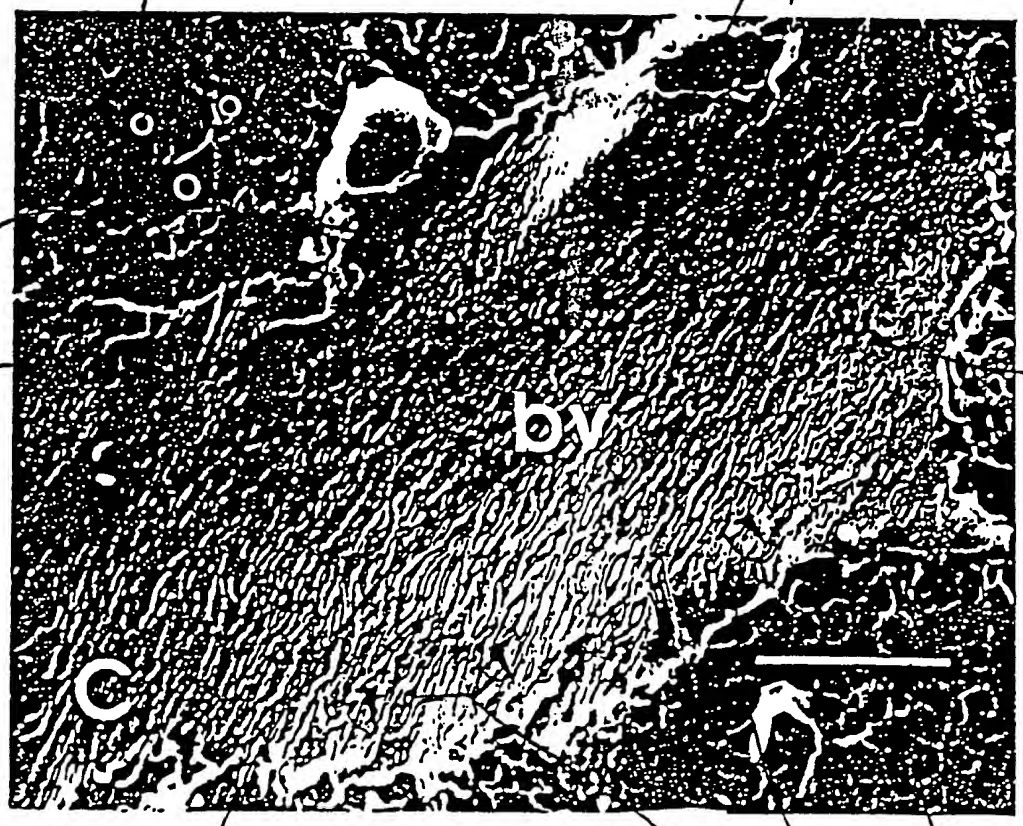


FIGURE 2 C

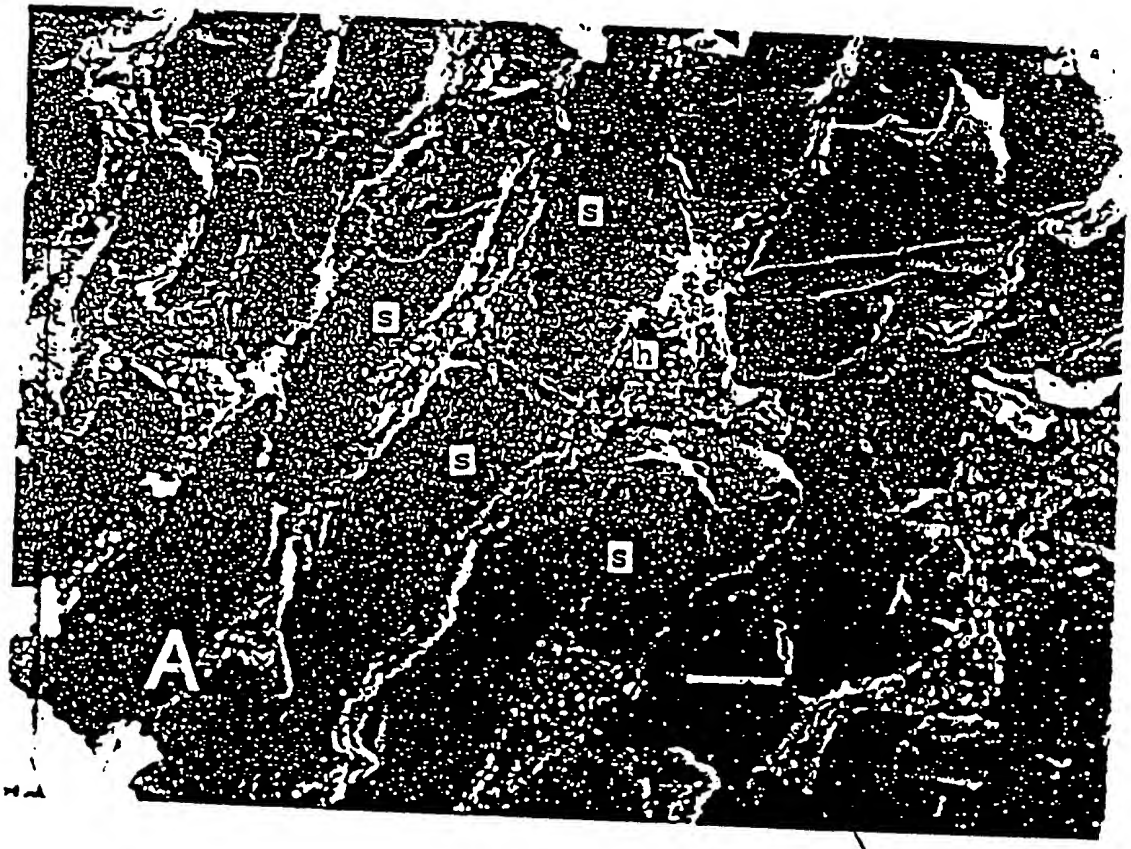


FIGURE 3A

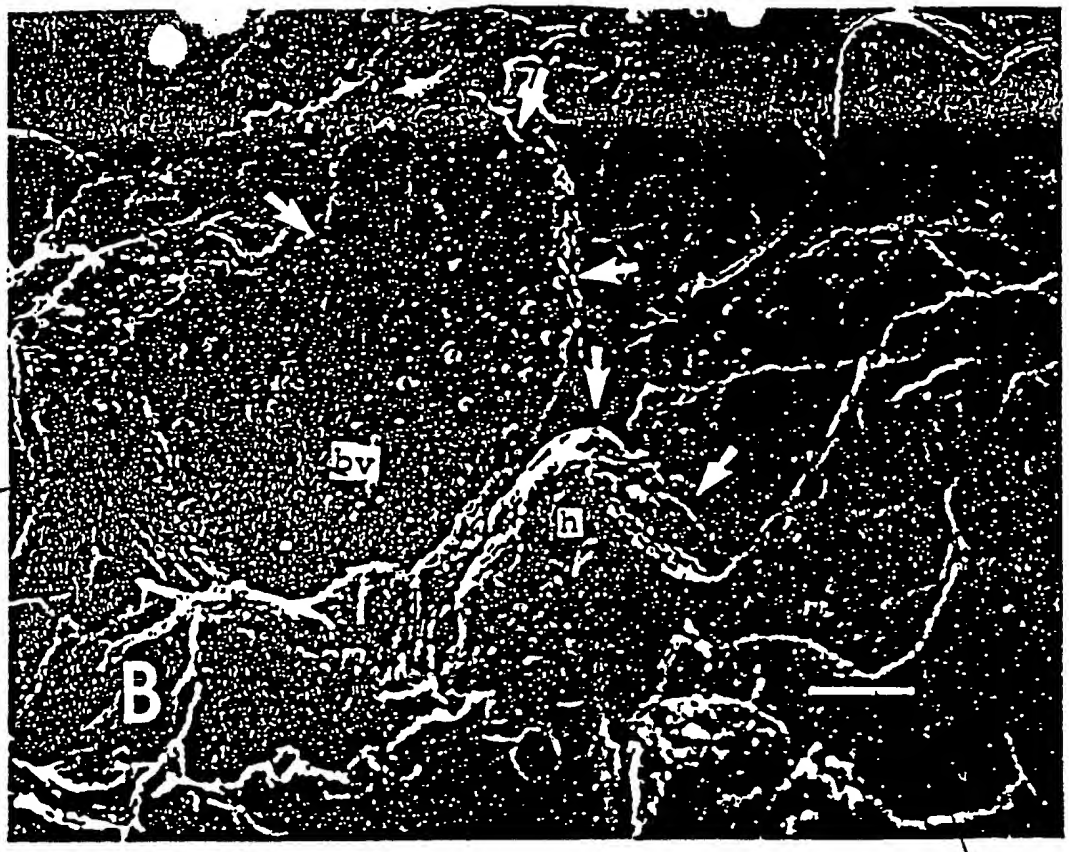


FIGURE 3 B

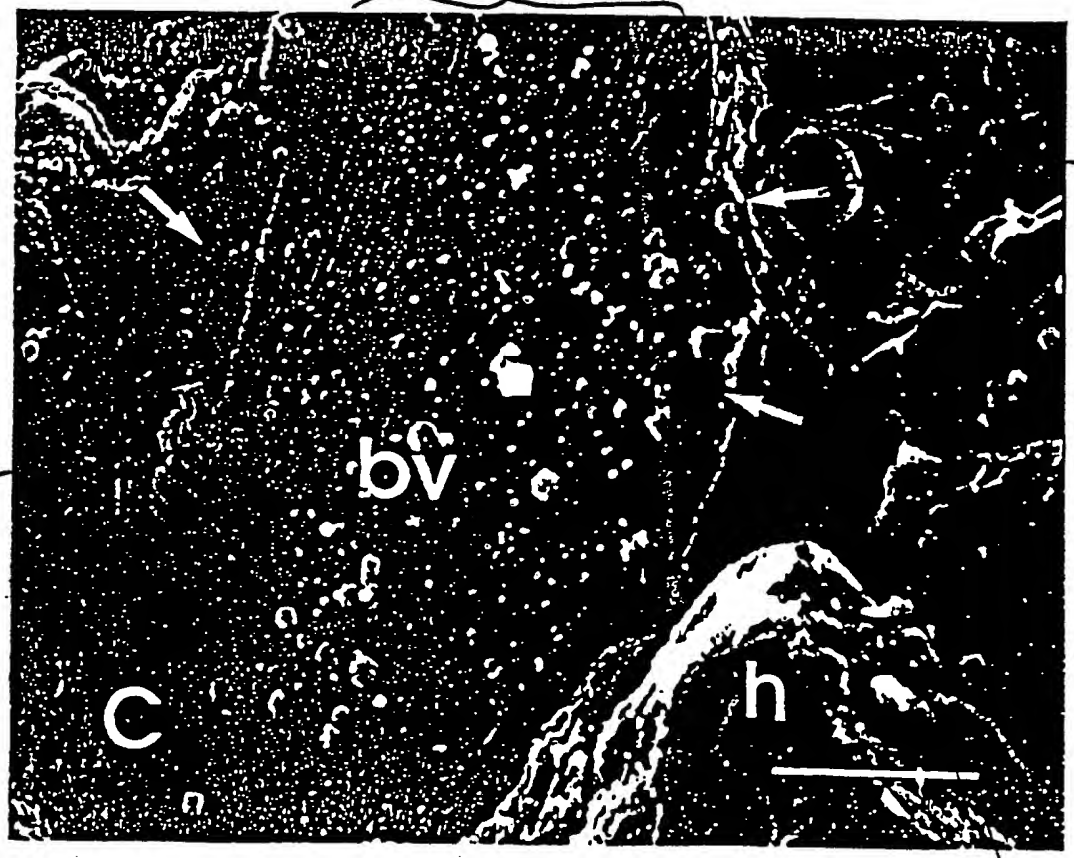


FIGURE 3C

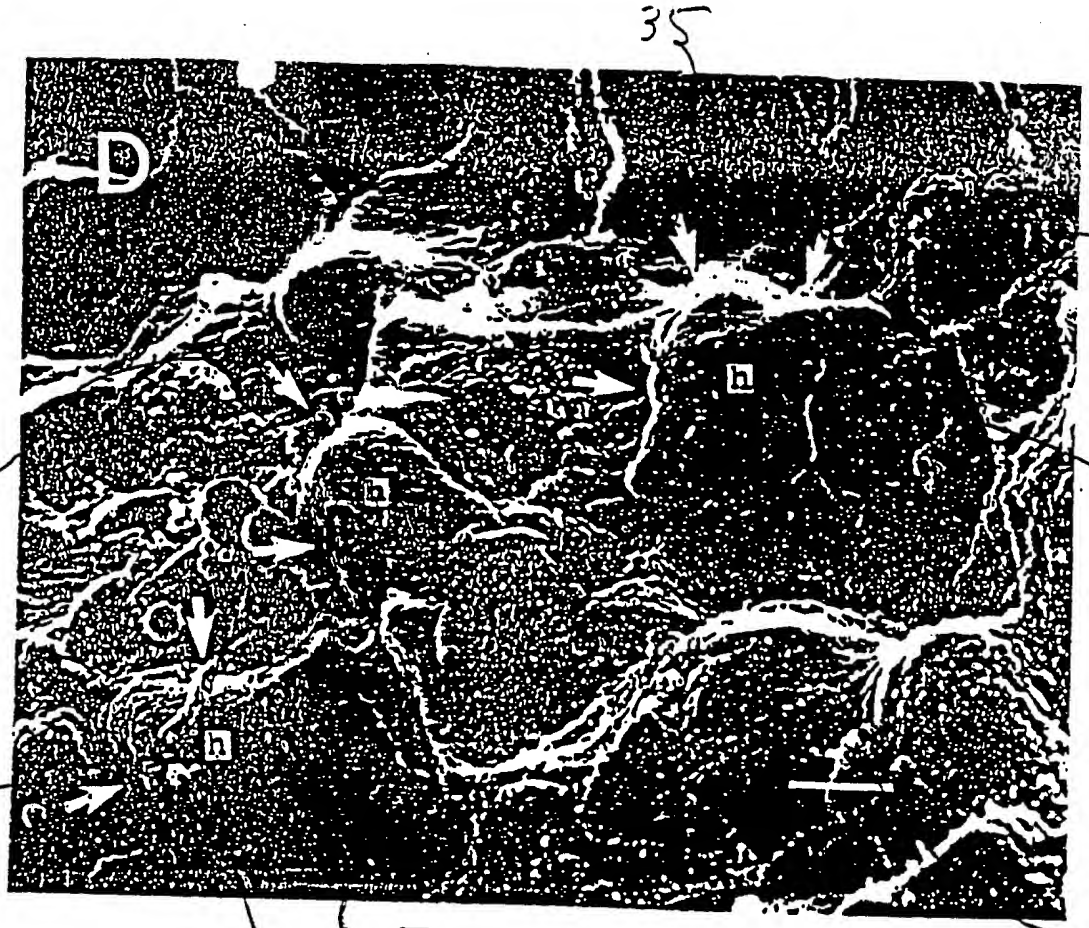


FIGURE 3D

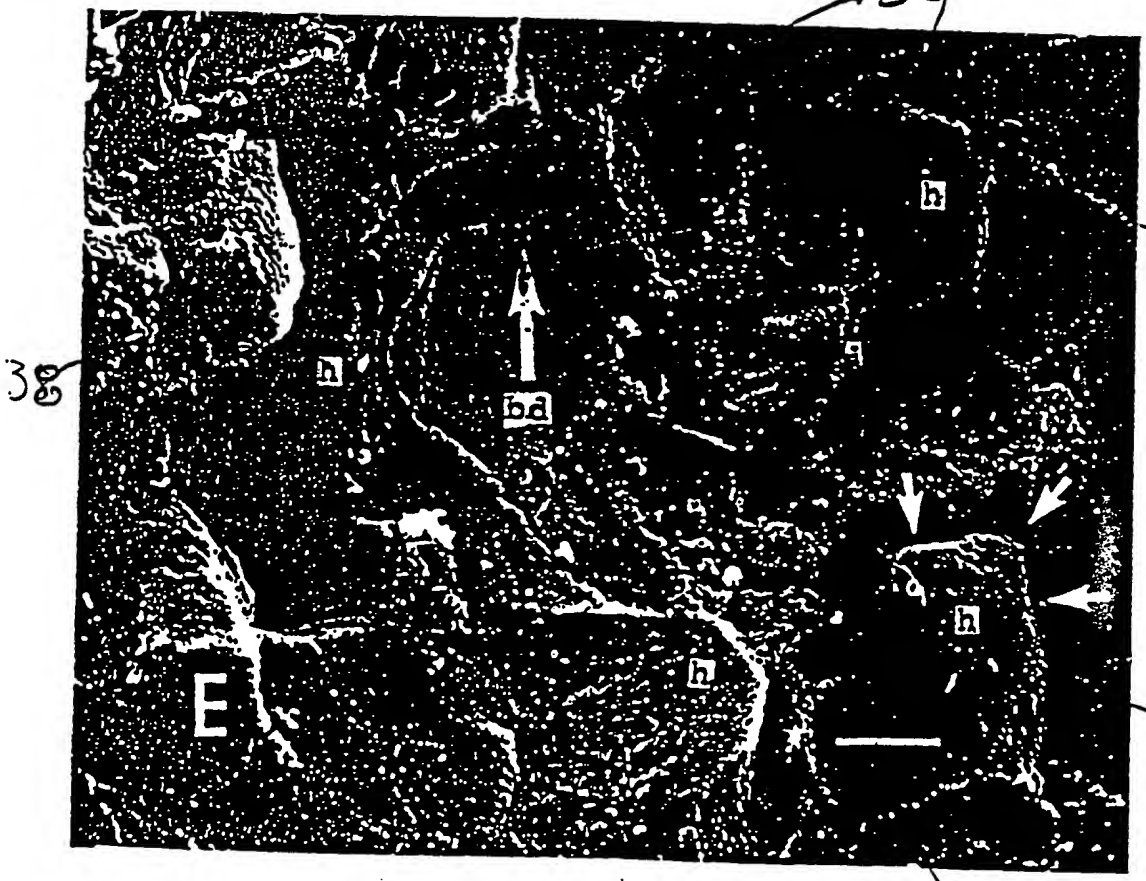


FIGURE 3 E

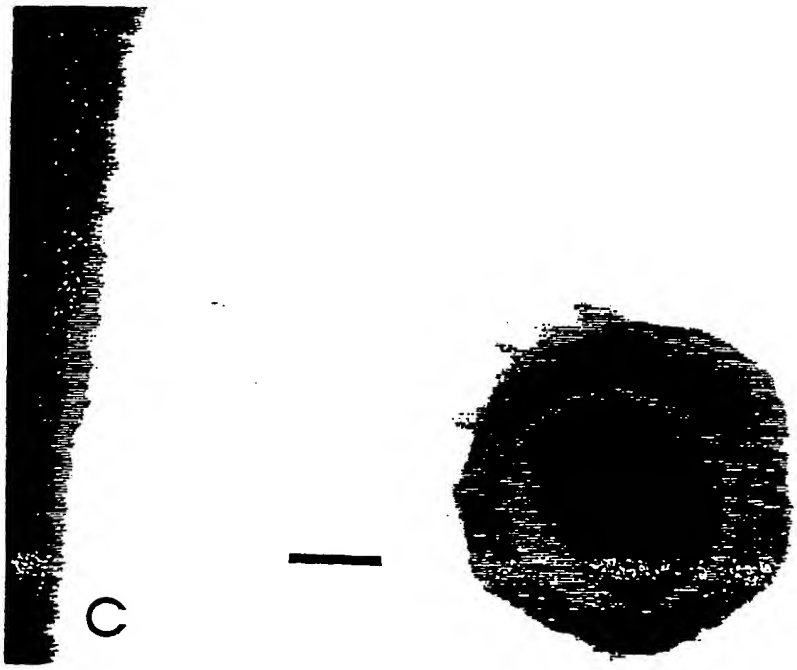


FIGURE 4A

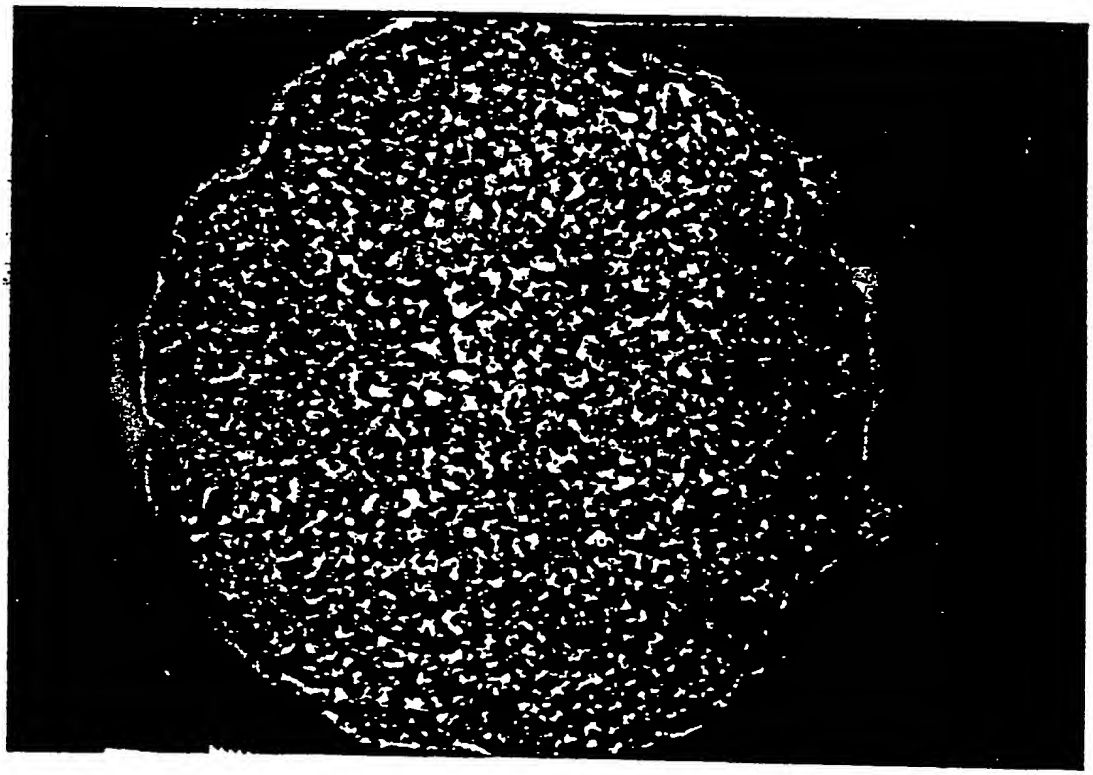


FIGURE 4B

FIGURE 4C

FIGURE 4D

FIGURE 7A

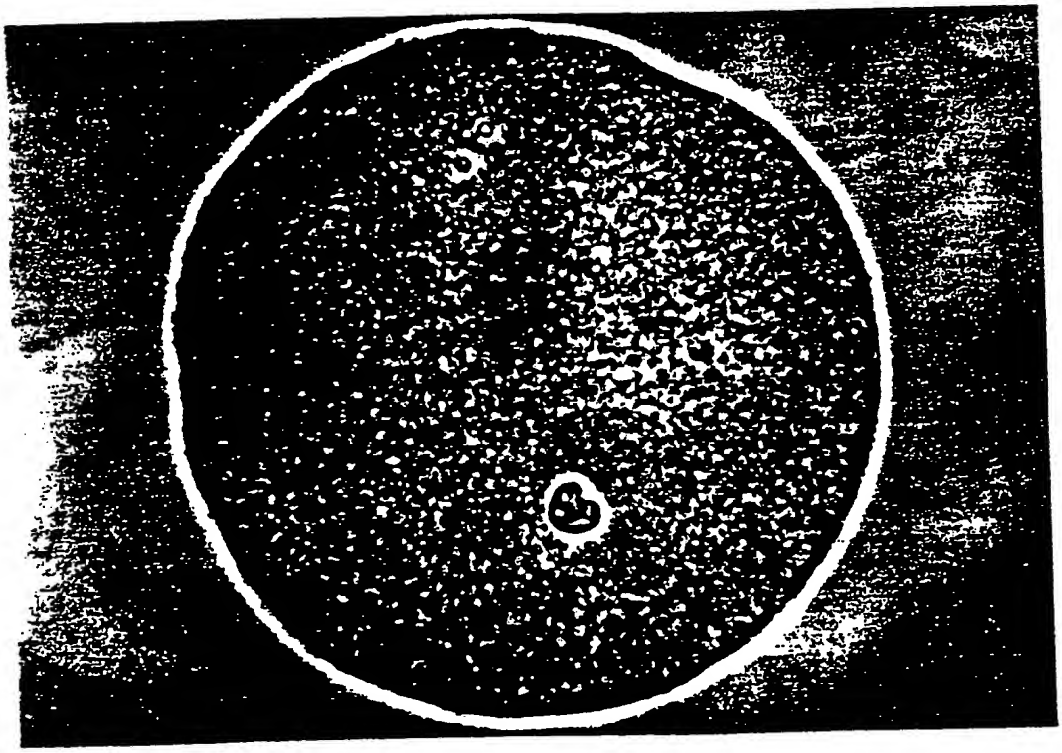


FIGURE 7B

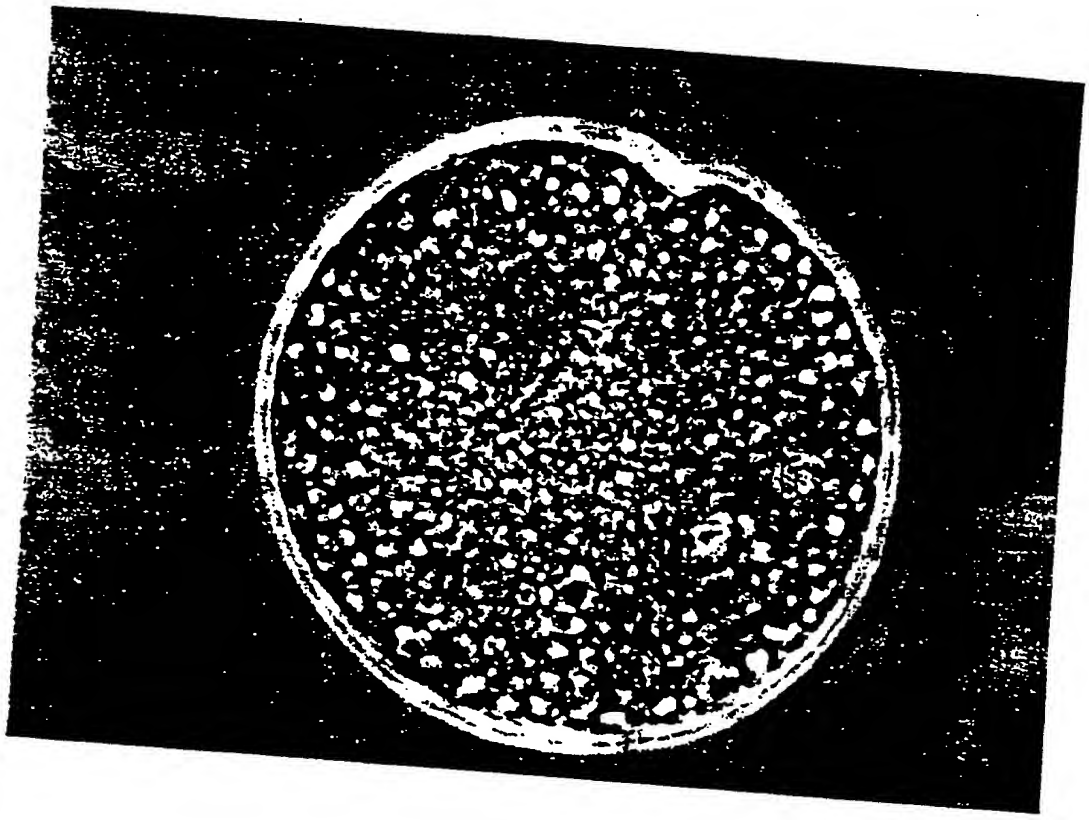


FIGURE 7C

FIGURE 8

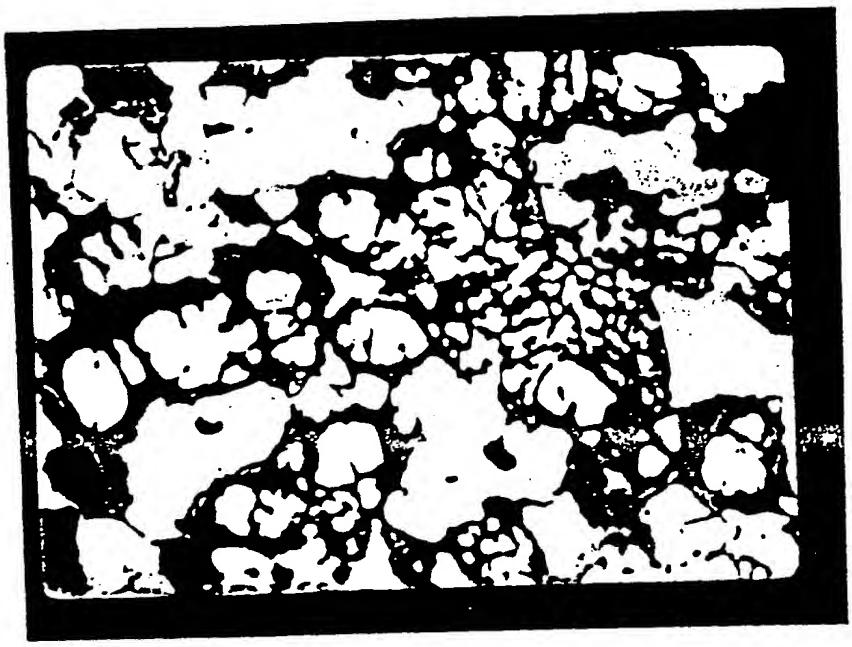


FIGURE 9

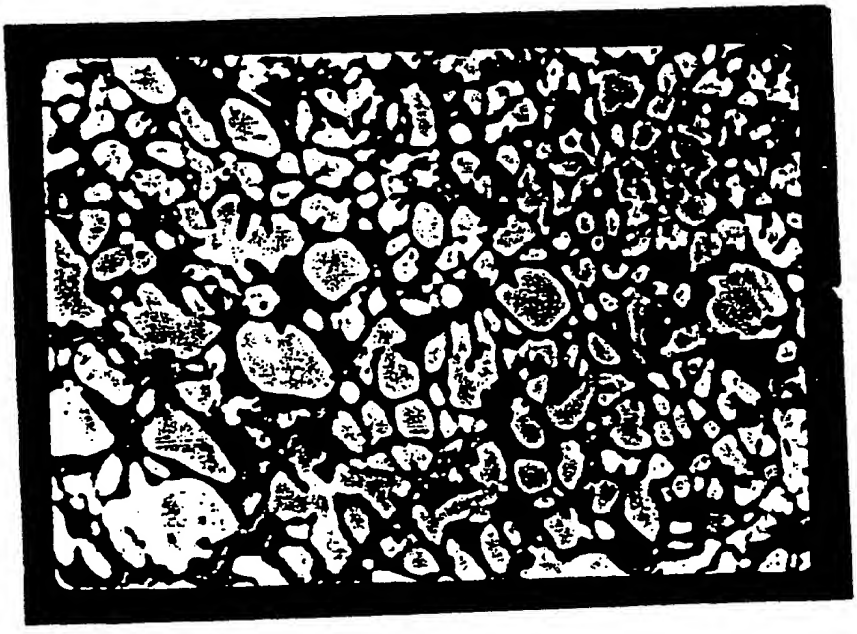


FIGURE 10

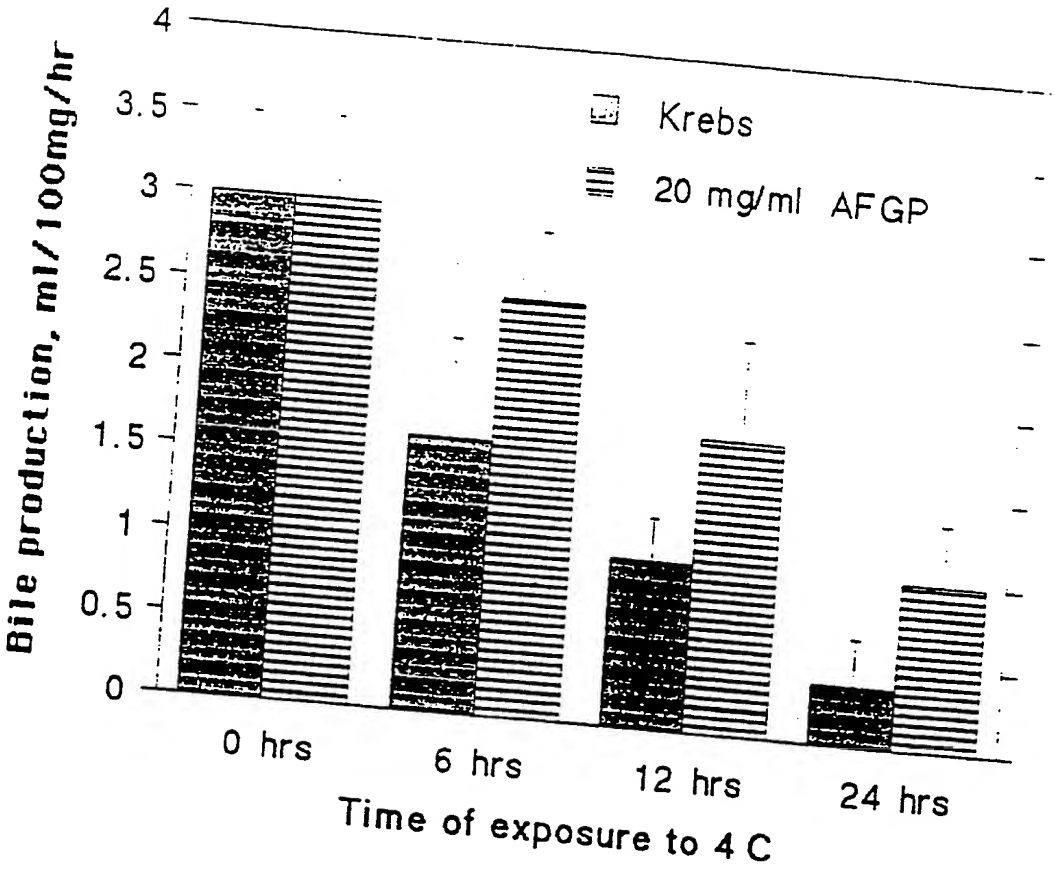
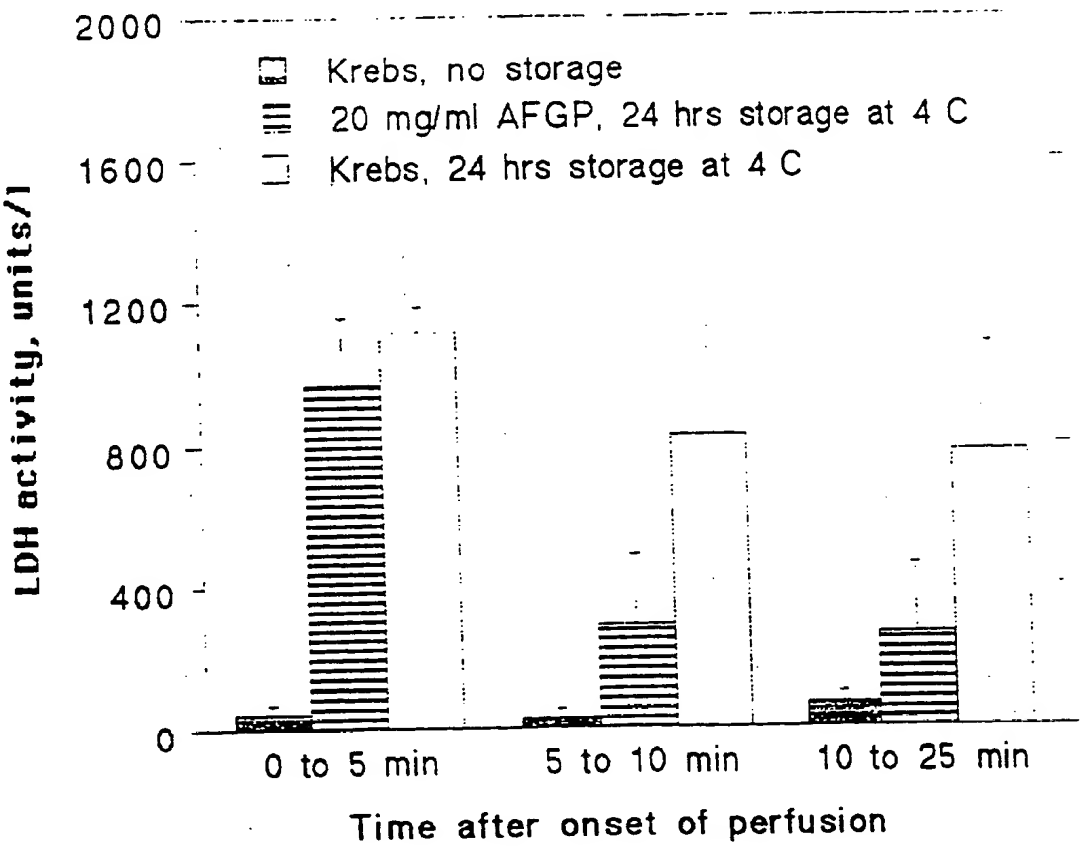

Bile production, ml/100mg/hr
4
3.5
3
2.5
2
1.5
1
0.5
0
Krebs
20 mg/ml AFGP
0 hrs
6 hrs
12 hrs
24 hrs
Time of exposure to 4 C

FIGURE 11


2000
1600
1200
800
400
0
LDH activity, units/l
Krebs, no storage
20 mg/ml AFGP, 24 hrs storage at 4 C
Krebs, 24 hrs storage at 4 C
0 to 5 min
5 to 10 min
10 to 25 min
Time after onset of perfusion

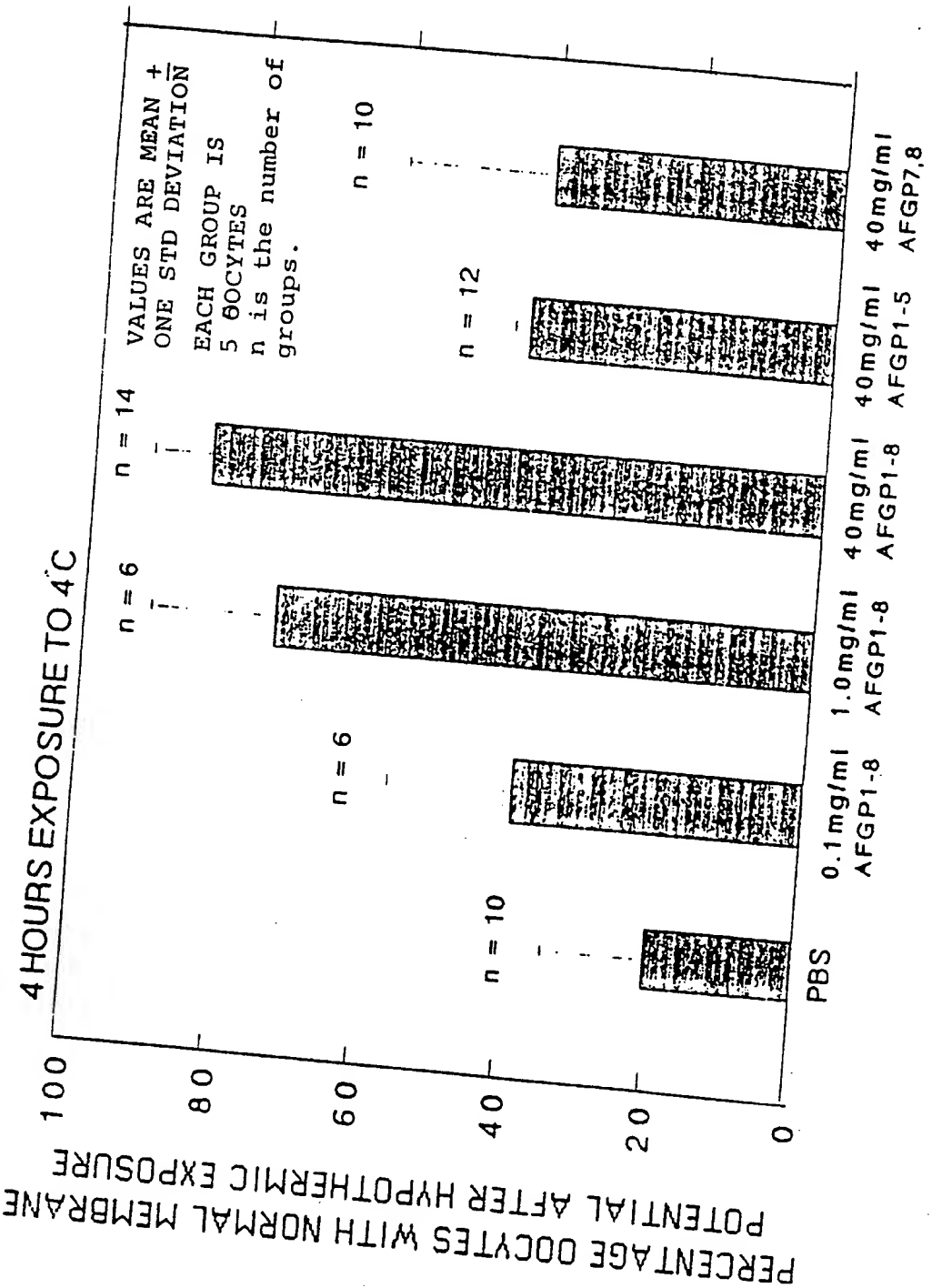

PERCENTAGE OOCYTES WITH NORMAL MEMBRANE POTENTIAL AFTER HYPOTHERMIC EXPOSURE
4 HOURS EXPOSURE TO 4°C
VALUES ARE MEAN ± ONE STD DEVIATION
EACH GROUP IS 5 OOCYTES
n is the number of groups.
n = 14
n = 6
n = 6
n = 10
n = 10
n = 12
0
20
40
60
80
100
PBS
0.1mg/ml AFGP1-8
1.0mg/ml AFGP1-8
40mg/ml AFGP1-8
40mg/ml AFGP1-5
40mg/ml AFGP7,8

FIGURE 12

LEGEND SEE FIG. 12

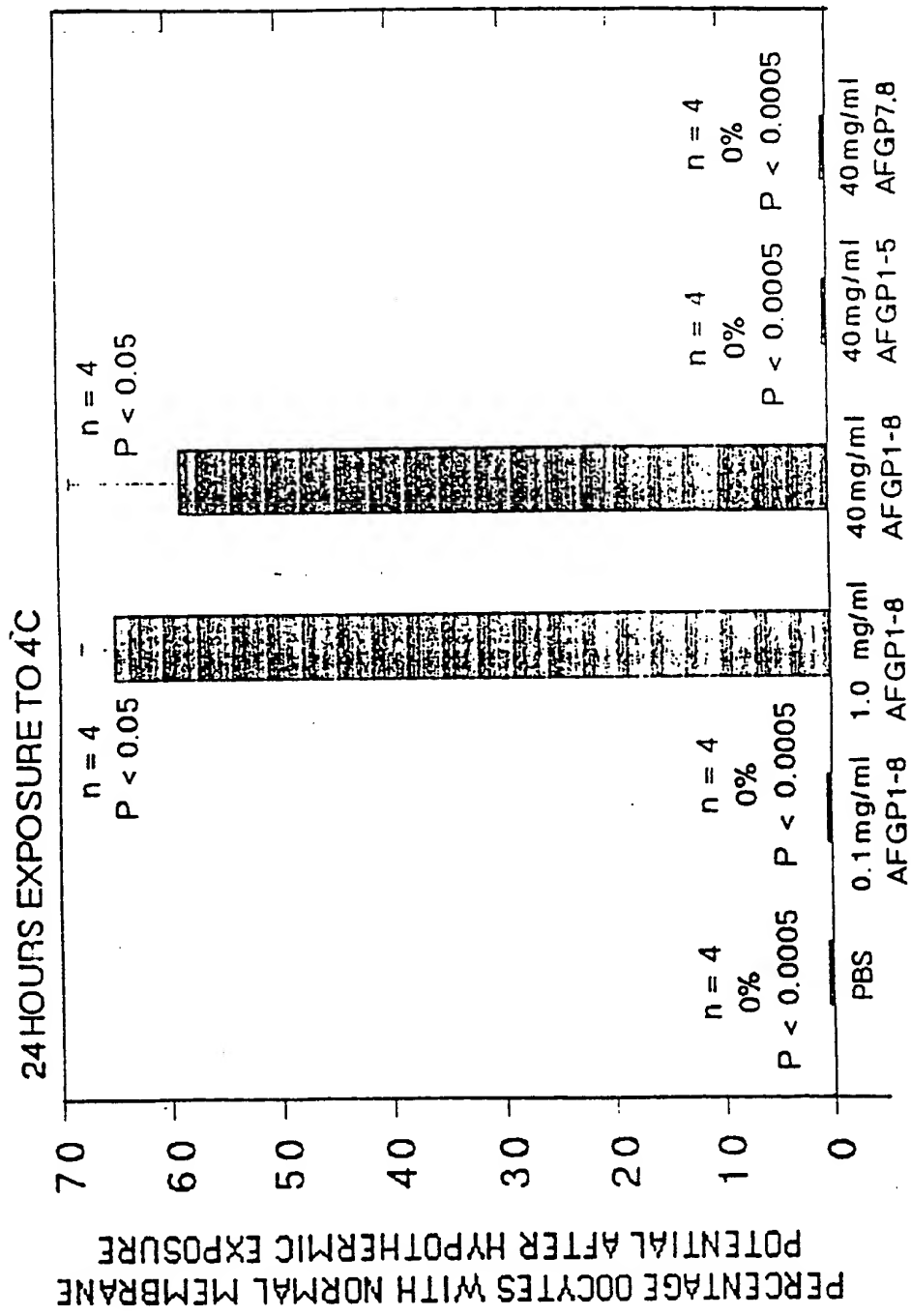


FIGURE 13

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 91/00351

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A 01 N 1/02
U.S. Cl: 435/1

II. FIELDS SEARCHED

Classification System	Minimum Documentation Searched 7
	Classification Symbols
U.S. Cl.	435/1, 62/64; 426/524

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

BIOSIS: "CRYOPRESERVZ OR CRYOPROTECT"
"POLYPEPTIDE OR GLYCOPOLYPEPTIDE"

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
	SEE ATTACHED	

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

25 APRIL 1991

Date of Mailing of this International Search Report

06 JUN 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

JANE WILLIAMS

Form PCT/ISA/210 (second sheet) (rev. 11-87)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers .. , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

I. Compositions: claims 1-8,11-40 and

Method 1. claims 9,10,43-46, Preserving cells, tissues

II. Method 2. claim 41, Preserving food

III. Method 3. claim 42, Treating disease

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4 ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest

☐ No protest accompanied the payment of additional search fees.

X/Y	Annual Review of Physiology, Volume 45, issued 1983, J Duman et al, "The Role of Hemolymph Proteins in the Cold Tolerance of Insects," pages 261-270, see page 261, paragraph 1, page 263, paragraph 1.	1/4-33, 36
X/Y	US, A, 4,059,967, (ROWE et al.) 29 November 1977, see abstract.	1-3, 30, 31, 39, 41-43/ 1-3, 30, 31, 39, 41-43
Y	US, A, 4,155,331 (LAWRENCE et al) 22 May 1979, see col 7, line 45-55.	9, 10
X	Science Volume 163, issued 07 March 1969, A.L. DeVries et al "Freezing Resistance in Some Antarctic Fishes," pages 1073-1075, see entire document.	6, 7, 32, 33, 34, 35
X/Y	Nature, Volume 333, issued 19 May 1988, D.S.C. Yang et al, "Crystal Structure of an Antifreeze Polypeptide and Its Mechanistic Implications," pages 232-237, see entire document.	37/37
X/Y	European Journal of Biochemistry, Volume 151, issued 1985, C.L. Hew et al, "Structures of Shorthorn Sculpin Antifreeze Polypeptides," pages 167-172, see entire document.	38/38
Y	US, A, 4,688,387, (CONAWAY) 25 August 1987, see col. 8, lines 30-42.	41, 42, 43-46

PCT Rules 13.1 and 13.2 do not provide for multiple distinct methods within a single general inventive concept.